(2)

AD____

PREVENTION OF PARALYTIC NEUROTOXIN ACTION ON VOLTAGE-SENSITIVE SODIUM CHANNELS

MIDTERM REPORT

WILLIAM A. CATTERALL

FEBRUARY 10, 1992

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-90-C-0115

University of Washington
Department of Pharmacology, SJ-30
Seattle, Washington 98195

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 10, 1992	Midterm 9/12/90 - 1/11/92

4. TITLE AND SUBTITLE
Prevention of Paralytic Neurotoxin Action on Voltage-Sensitive Sodium Channels

5. FUNDING NUMBERS
DAMD17-90-C-0115
61102A
3M161102S12 AA
DA335507

6. AUTHOR(S)
William A. Catterall

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Washington
Department of Pharmacology, SJ-30
Seattle, WA 98195

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS
Synthetic peptides; Neurotoxins; Sodium channels; Receptor sites; RA 1

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Contract No. DAMD17-90-C-0115

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI - Signature William Gatterall DATE 11/10/92

TABLE OF CONTENTS

INTRODUCTION

Voltage-sensitive sodium channels that generate action potentials in nerve and muscle are the targets of at least ten different classes of paralytic neurotoxins. Sodium channels have been purified to homogeneity from mammalian brain, their functional activities have been restored by reconstitution into phospholipid vesicles, and their principal subunit has been cloned, sequenced, and expressed. In previous work, the actions of neurotoxins on sodium channels have been studied using a combination of ion flux and whole cell patch voltage clamp techniques. Radioligand binding assays have been developed for each of the several neurotoxin receptor sites on the purified sodium channel from mammalian brain. Neurotoxin receptor site 3, which binds scorpion and sea anemone toxins, has been located to an extracellular peptide segment in domain I of the sodium channel α subunit. The experiments in this contract have the following objectives: 1. Synthesize peptides corresponding to segments of the sodium channel that form part of the receptor site for α-scorpion toxins, prepare polyclonal and monoclonal antibodies against them, and use these reagents to develop an effective treatment to prevent neurotoxicity of α-scorpion toxins and sea anemone toxins; 2. Covalently label additional neurotoxin receptor sites associated with voltage-sensitive sodium channels; 3. Prepare additional polyclonal site-directed anti-peptide antibodies against defined sequences of the rat brain sodium channel and determine their binding sites and functional effects; 4. Locate the covalently labeled neurotoxin receptor sites in the primary structure of the sodium channel using site-directed antibodies and protein microsequencing methods; and 5. Prevent toxin binding and action in vitro and in vivo by application of monoclonal antibodies against the peptide components of the toxin receptor sites or of synthetic peptides which mimic the peptide components of the toxin receptor site.

This Mid-Term Report summarizes the progress to date on our research contract to develop peptide and/or antibody reagents which prevent the actions of paralytic neurotoxins on voltage-sensitive sodium channels. The Report is organized under headings corresponding to each Task, and Experimental Procedures, Results, Discussion, and Figures illustrating data relevant to each Task are presented under each heading.

TASK I. INHIBITION OF ALPHA SCORPION TOXIN BINDING

Alpha scorpion toxins are basic polypeptides of approximately 70 amino acid residues which bind to neurotoxin receptor site 3 on the sodium channel and slow sodium channel inactivation. Using photoreactive scorpion toxin derivatives, we have previously covalently labeled a polypeptide component of this receptor site and determined that the site of covalent labeling is in the extracellular region of homologous domain I of the sodium channel α subunit (Fig. 1). The work under the current research contract has followed up those original observations by developing specific antibody and peptide reagents designed to block the access of the toxin molecule to its receptor site. Anti-peptide antibodies directed against peptide segments of the extracellular domain of the sodium channel were prepared and their ability to prevent toxin binding to the sodium channel was studied using radioligand binding methods. In addition, peptides were synthesized corresponding to amino acid sequences in the extracellular domains of the α subunit and tested for their ability to block the binding of α-scorpion toxins to sodium channels by interacting with the active site on the toxin. The effects of these reagents on the binding of α-scorpion toxins were studied for purified and reconstituted sodium channels, sodium channels in rat brain synaptosomes, and cloned sodium channels expressed in a clonal mammalian cell line.

Experimental Procedures

Materials. *Leiurus quinquestriatus* toxin V (LqTx) was purified from venom purchased from Sigma and was radiolabeled by lactoperoxidase catalyzed iodination as previously described (Catterall, 1977). Batrachotoxin (BTx) was a gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, NIH).

Anti-peptide antibodies. Synthetic peptides and antibodies used in this study were prepared using the method as described by Gordon et al. (1987). Peptides corresponding to residues of the rat brain Type IIA sodium channel sequences plus an N-terminal lysine extension were synthesized by solid phase method (Merrifield, 1963) and purified by reversed-phase HPLC. Antisera directed against synthetic peptides corresponding to different sequences of the α-subunit of the Type IIA sodium channel were prepared as described by Gordon et al (1987, 1988). A standard glutaraldehyde linkage of the amino groups of purified peptides to BSA was performed using the method of Orth (1979). The conjugate was dialyzed against phosphate-buffered saline and emulsified in an equal volume of Freund's complete adjuvant (initial injection) or incomplete adjuvant (subsequent boosts). The emulsion was injected subcutaneously into New Zealand White rabbits at 3-week intervals. Antisera were collected after the second injection, purified, and tested for antigen recognition by RIA. Identity of purified peptides was verified by amino acid analysis and determination of amino acid sequence. IgG was isolated from antisera by protein A-Sepharose chromatography. Fab fragments were generated from IgG using immobilized papain and were resolved from Fc fragments and unreacted IgG by protein A-Sepharose chromatography. All antibodies recognize the peptide antigen in ELISA assays carried out as described by De Jongh et al (1989, 1991) and bind to native sodium channels in immunoprecipitation assays carried out as described by Costa and Catterall (1984) and Gordon et al (1988).

^{125}I-LqTx Binding to Purified and Reconstituted Sodium Channel. Rat brain sodium channels purified through the step of chromatograpy on wheat germ agglutinin-Sepharose (Hartshorne and Catterall, 1984) at concentrations of 300-400 pmol/ml in a solution containing 25 mM Hepes-Tris (pH 7.4), 100 mM Na_2SO_4, 0.4 mM $MgSO_4$, 157 mM N-acetylglucosamine, 4 μM tetrodotoxin, 1.65% Triton X-100, 0.19% phosphatidylcholine, and 0.12% phosphatidylethanolamine were reconstituted in phospholipid vesicles as previously described (Tamkun et al, 1984; Feller et al, 1985). After preincubation of reconstituted vesicles with preimmune IgG, site-directed antibody, or monoclonal antibody, ^{125}I-LqTx binding assays were performed essentially as described previously (Tamkun et al, 1984). Assays were initiated by the addition of 40 μL of preincubation mixture to 160 μL of a solution containing 0.23 nM ^{125}I-LqTx, 105 mM Tris-SO_4, 0.5 mM $MgSO_4$, 25 mM Hepes-Tris, 150 mM sucrose, and 4 mg/ml BSA, pH 7.4. The sucrose concentration was adjusted to maintain osmolarity equal to the intravesicular medium. The reaction mixture was then incubated for five minutes at 37°C, diluted with 3 ml of wash buffer containing 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1 mg/ml BSA, and sucrose sufficient to maintain osmolarity, filtered over GF/F filters under vacuum pressure, and washed three more times with the same wash buffer. Nonspecific toxin binding was determined in the presence of 1 μM unlabeled LqTx and accounted for 20-40% of the total binding. Approximately 75% of the nonspecific binding is to GF/F filters alone.

^{125}I-LqTx Binding to Rat Brain Synaptosomes. Rat brain synaptosomes were prepared as previously described (Thomsen and Catterall, 1989) and stored at -80°C until used. After preincubation of synaptosomes with IgG as described in each figure legend, ^{125}I-LqTx binding was quantitated as described for reconstituted sodium channel. Nonspecific binding was determined in the presence of 1 μM LqTx and accounted for 10-25% of the total binding.

^{125}I-LqTx Binding to CNaIIA-1 cells. Scorpion toxin binding to CNaIIA-1 cells was measured by a modification of the method of Catterall (1977) as described by West et al (1992).

Results

Inhibition of binding of ^{125}I-LqTx by site-directed antibodies. We began these studies with a set of site-directed anti-peptide antibodies that had been developed in conjunction with localization of the site of covalent labeling of the sodium channel by photoreactive derivatives of LqTx (Tejedor and Catterall, 1988). This previous work resulted in the identification of a region in the extracellular loop between transmembrane segments S5 and S6 of domain I as a component of the scorpion toxin binding site (Fig. 1A). Six site-directed antibodies

(IgG) that recognize different proposed extracellular regions of the Type IIA sodium channel α-subunit were evaluated for their ability to inhibit ^{125}I-LqTx binding to sodium channels in reconstituted phospholipid vesicles and synaptosomes (Fig. 1B). Preincubation of purified sodium channels reconstituted in phospholipid vesicles with three of the six antibodies, anti-SP28, anti-SP31, and anti-SP29, inhibited ^{125}I-LqTx binding by 28%, 55%, and 54%, respectively (Fig. 2A). Similar levels of antibody-mediated inhibition of ^{125}I-LqTx binding were observed when partially purified sodium channel was preincubated for four hours at 4°C with the same quantity of these antibodies prior to reconstitution and toxin binding assays. Anti-SP8, anti-SP14, and anti-SP13 did not have significant effects on LqTx binding (Fig. 2A). Anti-SP19, an antibody recognizing the proposed intracellular segment of the α-subunit between homologous domains III and IV associated with sodium channel inactivation (Gordon et al, 1988), also had no effect on toxin binding.

Similar results were observed using rat brain synaptosomes (Fig. 2B). In this case, anti-SP31, anti-SP28, and anti-SP29 inhibited ^{125}I-LqTx binding by 33%, 29%, and 27%, respectively, while the other three antibodies had no effect.

Anti-SP31 and anti-SP28 are directed against synthetic peptides corresponding to amino acid residues which are located on the proposed extracellular loop between transmembrane segments S5 and S6 of homologous domain I near the site previously identified by covalent attachment of LqTx derivatives (Fig. 1, Tejedor and Catterall, 1988). Anti-SP29 is directed against a synthetic peptide corresponding to amino acid residues located on the proposed extracellular segment between transmembrane segments S5 and S6 in homologous domain IV. Since these three antibodies inhibit LqTx binding while other closely related anti-peptide antibodies do not, our results suggest that the corresponding extracellular segments of domains I and IV interact in forming the receptor site for LqTx.

Concentration-effect curves for inhibition of LqTx binding to either reconstituted rat brain sodium channel or synaptosomes by anti-SP8 indicate that inhibition is concentration-dependent (Fig. 3). Using reconstituted vesicles, EC_{50} values of 0.51+/- 0.21 μM, 0.39+/-0.12 μM, and 0.57+/-0.20 μM and maximal inhibition of 39%, 36%, and 47% were obtained for anti-SP31, anti-SP28, and anti-SP29, respectively (Fig. 3A). Higher EC_{50} values of 0.65+/-0.15 μM, 2.0+/-0.66 μM, and 1.35+/-0.38 μM and similar maximal levels of inhibition of 29%, 43%, and 33% were observed using rat brain synaptosome preparations (Fig. 3B). It should be noted that the concentrations of antibody listed correspond to total IgG isolated from antisera and therefore estimated EC_{50} values substantially exceed the true values for the active antibodies. Definition of maximal levels of inhibition was difficult because of limitations in the amount of antibody that could be added during the preincubation period.

The extent of inhibition of LqTx binding by site-directed antibodies was not increased by treatments designed to improve their access to their binding sites. Neuraminidase treatment of purified sodium channel to remove sialic acid residues prior to reconstitution did not result in enhanced inhibition of toxin binding, and smaller Fab fragments generated by papain treatment of IgG produced similar levels of inhibition of ^{125}I-LqTx binding to reconstituted vesicles as native IgG. Moreover, the effects of maximal concentrations of anti-SP31, anti-SP28, and anti-SP29 were not additive. These results suggested that the anti-peptide antibodies completely inhibit LqTx binding to sodium channels to which they can gain access and bind, but that a fraction of sodium channels do not bind the anti-peptide antibodies.

In order to develop antibodies which might inhibit LqTx binding more completely at lower concentrations, peptides SP33, SP34, SP35, SP36, SP37, SP38, SP41, SP42, and SP43 representing overlapping segments of the extracellular regions of domains I through IV (Fig. 1B) were synthesized as described under Experimental Procedures. Except for SP42, these were purified by reversed phase HPLC, coupled to bovine serum albumin, and used for production of polyclonal anti-peptide antisera. SP42 was insoluble in aqueous solvents. Therefore, a new nonaqueous solvent-based protocol for direct coupling to bovine serum albumin was developed and used for this peptide as described under Experimental Procedures. Antisera were assayed by

ELISA against the peptide antigen to follow the progress of the immunization process. In general, usable titers of greater than 1:1000 were obtained only following the second or the third booster injection. Titers in radioimmune assays for immunoprecipitation of sodium channels or in the ELISA assay against the antigenic peptide for the most current antisera collections are illustrated in Fig. 4 and compared to results for previously developed antibodies against synthetic peptides SP8, SP13, and SP19. IgG fractions were purified by chromatography on protein A-Sepharose and, in some cases, immunoreactive antibodies were further purified by affinity chromatography on peptide antigen immobilized on Sepharose beads. We are still in the process of testing these new antibodies for inhibition of high affinity binding of LqTx to synaptosomes and transfected cells expressing Type IIA sodium channels using similar methods to those described above.

Inhibition of α-scorpion toxin binding by sodium channel peptides. Antibodies are bulky reagents which may have difficulty in reaching their binding sites on sodium channels. This reduced access may prevent complete inhibition of toxin binding by these reagents if the key determinants of toxin binding are protected from antibody interaction. One potential approach to circumvent this problem is to use sodium channel peptides that represent a portion of the receptor site for the toxin to block the active site on the toxin molecule and prevent its binding. This approach may be restricted to macromolecular toxins which themselves have a distinct active site for binding to their target, as has been demonstrated for the α-scorpion toxins (El Ayeb et al, 1986). Peptides of 16 to 20 residues were synthesized as described under Experimental Procedures and purified by reversed phase HPLC. These peptides were pre-incubated with ^{125}I-labeled α-scorpion toxin to allow interaction with the toxin. The mixture was then added to synaptosomes and specific binding of the scorpion toxin to the sodium channels in the synaptosomes was measured. A range of peptide concentrations and toxin concentrations was studied, but the results are illustrated in Fig. 5 for a single (100 μM) concentration of peptide. Three peptides reduced α-scorpion toxin binding in this series of experiments: SP38, SP41, and SP42. The effects of SP38 and SP41 were significant at $p<0.05$; they were more consistent than SP42 whose effects did not reach statistical significance.

For some peptides, the level of α-scorpion toxin binding was increased after preincubation with the toxin. This was a surprising result so we examined whether the peptides could increase the nonspecific binding of α-scorpion toxin to the glass fiber filters used in the synaptosome binding assay. We found that some peptides did indeed have this effect. To circumvent this potential problem, we developed a different assay to measure peptide effects on scorpion toxin binding. This assay took advantage of the CNaIIA-1 cells which express Type IIA sodium channels (West et al, 1992). Since these cells are tightly adherent to the bottom of the culture dish, toxin and peptides can be incubated with the cells in the culture dish and binding can be measured by washing away the unbound toxin and determining the radioactivity in the cell extract. Using this assay, we also found that peptides SP38 and SP41 reduced α-scorpion toxin binding, but their effect was no greater than in the assay using synaptosomes (Fig. 6). Mixtures of the two peptides were only slightly more effective than the individual compounds alone and this difference did not achieve statistical significance. Mixtures of multiple peptides were less effective. For example, combinations of SP8, SP28, SP31, SP38, and SP41 gave little inhibition of scorpion toxin binding, perhaps because the peptides complex with or compete with each other reducing the interaction of the effective peptides with the toxin (Fig. 6, last column).

Overall, the experiments with peptides give results similar to the experiments with our initial set of antibodies. Both classes of reagents cause small reductions in binding of α-scorpion toxins if the reagents are directed toward sequences in the extracellular loop of domain I of the sodium channel. However, the incomplete inhibition indicates that these sequences are adjacent to the toxin receptor site rather than directly within it so that access is reduced while high affinity binding is not completely prevented. Hopefully, one or more of the new set of anti-peptide antibodies that is currently being analyzed will provide a better inhibition of toxin binding.

Discussion

Interaction of extracellular segments of domains I and IV in forming the α-scorpion toxin receptor site. Photoreactive derivatives of LqTx were previously shown to be incorporated into the extracellular segment of the sodium channel between amino acid residues 317 and 400 in domain I (Tejedor and Catterall, 1988). Subsequent work shows that antipeptide antibodies recognizing residues 355-371 and 382-400 are partial inhibitors of LqTx binding while antibodies recognizing the adjacent residues 317-335 and several other sodium channel segments are not. These results implicate the peptide segment from residues 355 to 400, immediately adjacent to proposed transmembrane segment S6, as being located near, and possibly within, the α-scorpion toxin binding site.

Binding of α-scorpion toxins to the sodium channel is conformationally dependent (Catterall, 1977), and it was predicted (Tejedor and Catterall, 1988) that multiple polypeptide segments from different regions of the primary structure might contribute to formation of neurotoxin receptor site 3. Our results show that anti-SP29 is also a partial inhibitor of LqTx binding while several other anti-peptide antibodies including the nearby anti-SP13 are not. Amino acid residues 1686-1703 are located in the extracellular segment between proposed transmembrane segments S5 and S6 of domain IV near the junction with S5. Thus, it is our working hypothesis that amino acid sequences from these two distant regions of the primary structure of the sodium channel α subunit participate in formation of neurotoxin receptor site 3.

Although domains I and IV of the sodium channel α-subunit are distant from each other in the primary structure of the channel, the four homologous domains defined in the primary structure are proposed to be organized in a square array around a central transmembrane pore placing domains I and IV adjacent to each other in the three dimensional structure of the channel protein. Our results provide support for this widely accepted proposal. Scorpion toxins are small elliptical molecules of approximately 50x40x30 A. Their binding surface is thought to extend over part of one face of the molecule. The inhibition of LqTx binding by anti-peptide and monoclonal antibodies described here indicates that this small binding face may interact with a receptor site formed by the close apposition of residues in domain I and domain IV of the sodium channel α subunit. Further probing of these regions with anti-peptide antibodies is likely to reveal the peptide segments which are essential for this binding interaction and provide an avenue to prevention of toxin binding and action.

Future plans. It is disappointing that none of the peptide or antibody reagents developed to date are high affinity inhibitors of scorpion toxin binding. The results so far suggest that the anti-peptide antibodies bind near, but probably not directly within the α-scorpion toxin receptor site. We plan to analyze the new antibodies for inhibition of toxin binding and to use epitope-selection methods (De Jongh et al., 1991) to purify antibodies against shorter peptide segments to focus in on the toxin binding site.

Figures

FIG. 1. Overall structure and amino acid sequences of the extracellular segments of the α subunit. A. The transmembrane folding model of the α subunit of the sodium channel is illustrated. The locations of specific functional sites are indicated as follows: **P**, phosphorylation sites for cAMP-dependent protein kinase; **Y**, sites of binding of intracellularly directed antibodies; filled rectangles, sites of binding of extracellularly directed antibodies that inhibit α-scorpion toxin binding; Ψ, known sites of N-linked glycosylation; and ScTx, site of covalent labeling of the sodium channel by α-scorpion toxin derivatives. **B.** Amino acid sequences of the major extracellular loops in domains I through IV and synthetic peptides (SPxx) which correspond to them.

FIG. 2. Inhibition of α-scorpion toxin binding to sodium channels in reconstituted phospholipid vesicles and synaptosomes by anti-peptide antibodies. A. Sodium channels in reconstituted phospholipid vesicles were preincubated with 5.5 μM preimmune IgG or 5.5 μM of the indicated site-directed antibodies (IgG) in a total volume of 300 μl containing 50 mM NaCl, 3 mM Hepes-Tris, 133 mM glycine HCl, pH 7.4, and 1 μM BTx for four hours at 4°C with rotation, and ^{125}I-LqTx binding was measured as described under Experimental Procedures. **B.** Rat brain synaptosomes (50 μl of a 25 mg/ml suspension) were preincubated with 5.5 μM preimmune IgG or 5.5 μM of the indicated site-directed antibodies, in a total volume of 300 μl of 25 mM NaCl, 2 mM Hepes-Tris, 133 mM glycine HCl, pH 7.4, and 1 μM BTx, and ^{125}I-LqTx binding was measured as described under Experimental Procedures. The data are expressed as the percentage inhibition as compared to control samples having only preimmune IgG.

FIG. 3. Concentration dependence of inhibition of α-scorpion toxin binding to sodium channels in reconstituted phospholipid vesicles and synaptosomes by three active anti-peptide antibodies. A. *Inhibition of* ^{125}I-LqTx binding to sodium channels reconstituted in phospholipid vesicles by anti-SP28 (circles), anti-SP29 (squares), and anti-SP31 (triangles). Sodium channels in reconstituted phospholipid vesicles (100 μl) were preincubated for four hours with the indicated concentrations of site-directed antibodies, 2 μM preimmune IgG, and 1 μM BTx, in a total volume of 300 μl containing 50 mM NaCl, 3 mM Hepes-Tris, 133 mM glycine HCl, pH 7.4, and 1 μM BTx with rotation, and ^{125}I-LqTx binding was measured as described under Experimental Procedures.. The percentage inhibition at each antibody concentration was calculated from controls that contained only preimmune IgG. Each data point represents the mean of three to five independent experiments. **B.** Inhibition of ^{125}I-LqTx binding to rat brain synaptosomes by anti-SP28 (circles), anti-SP29 (squares), and anti-SP31 (triangles). Synaptosomes (50 μl of 25 mg/ml) were preincubated with 7 μM preimmune IgG and the indicated concentrations of site-directed antibodies in a total volume of 300 μl of 25 mM NaCl, 2 mM Hepes-Tris, 133 mM glycine HCl, pH 7.4, and 1 μM BTx, and ^{125}I-LqTx binding was measured as described under Experimental Procedures. Each data point represents the mean +/- SEM of three to four separate experiments in which triplicate determinations were made at each antibody concentration.

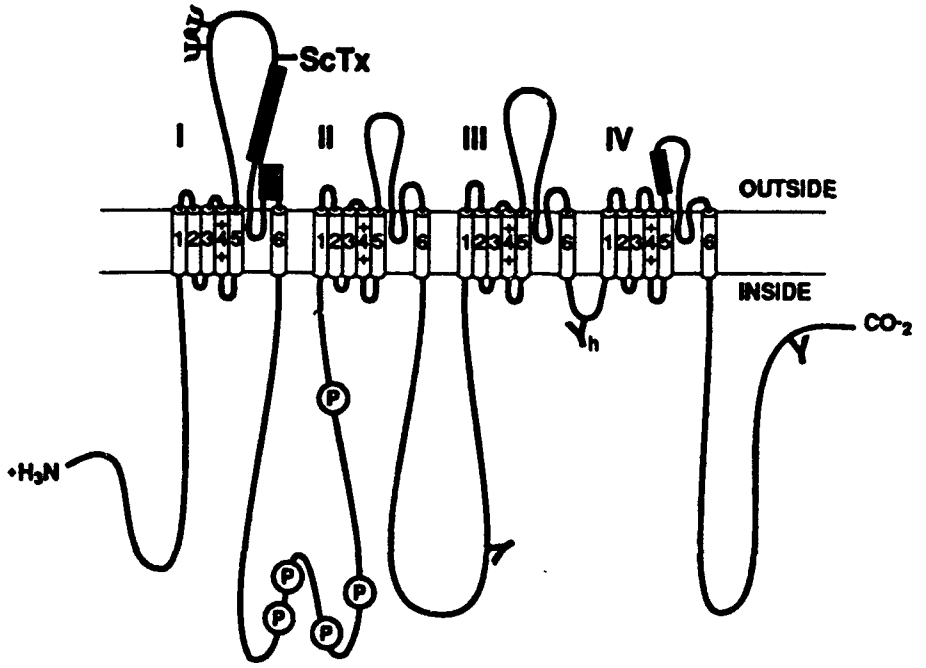
FIG. 4. ELISA analysis of new anti-peptide antibodies. Antisera against the indicated synthetic peptides were diluted to the titers listed on the abscissa, incubated in multiwell plates coated with synthetic peptide, and the bound antibody was measured by extensive washing and development in an enzyme-linked colorimetric alkaline phosphatase reaction as described under Experimental Procedures.

FIG. 5. Effect of synthetic peptides on binding of α-scorpion toxins to sodium channels in synaptosomes. ^{125}I-labeled α-LqTx was incubated with the indicated synthetic peptides for 60 min at 37°C. Synaptosomes were added and the incubation was continued for 30 min at 37°C. The synaptosomes and bound α-LqTx were then collected by vacuum filtration and washed as described under Experimental Procedures. Each point represents the average of three independent experiments.

FIG. 6. Effect of synthetic peptides on binding of α-scorpion toxins to sodium channels in CNaIIA-1 cells. ^{125}I-labeled α-LqTx was incubated with the indicated synthetic peptides for 60 min at 37°C. Sodium channels in reconstituted vesicles were added and the incubation was continued for 30 min at 37°C. The reconstituted vesicles and bound α-LqTx were then collected by vacuum filtration and washed as described under Experimental Procedures. Each point represents the average of three independent experiments.

FIGURE 1

A.



B.

DOMAIN I, CONSERVED SEGMENT

```
   --  --+       -   -       -     -     + +          -            +     - -       + +
FNWDEYIEDK SHFYFLEGQN DALLCGNSSD AGQCPEGYIC VKAGRNPNYG YTSFDTFSWA FLSLFRLMTQ DFWENLYQLT LRAAGKTYMI
          _____SP8_____ _____SP38____                                  _____SP28_____
                 _____SP41_____  _____SP31_____
```

DOMAIN II

```
                                  -  +   -   -  +     -          +     -  -   -  -
                          GKSYK ECVCKISNDC ELPRWHMHDF FHSFLIVFRV LCGEWIETMW DCMEVAGQTM
                          _____SP35_____     _____SP22_____
                                                  _____SP42_____
```

DOMAIN III

```
 +          - -       -     -    + + +   -                    +   -       - +   -   + --
 GKFYHCI NYTTGEMFDV SVVNNYSECQ ALIESNQTAR WKNVKVNFDN VGLGYLSLLQ VATFKGWMDI MYAAVDSRNV ELQPKYEDNL
        _____SP36_____          _____SP33_____           _____ SP14_____
                  _____SP43_____
```

DOMAIN IV

```
      ++-   --     -                                  - - -+-       + -
      KREVGIDDMFNF ETFGNSMICL FQITTSAGWD GLLAPILNSG ...PPDCDPEKDH PGSSVKGDCG NPSV
      _____SP29_____     _____SP34_____     _____SP13_____
```

FIGURE 2.

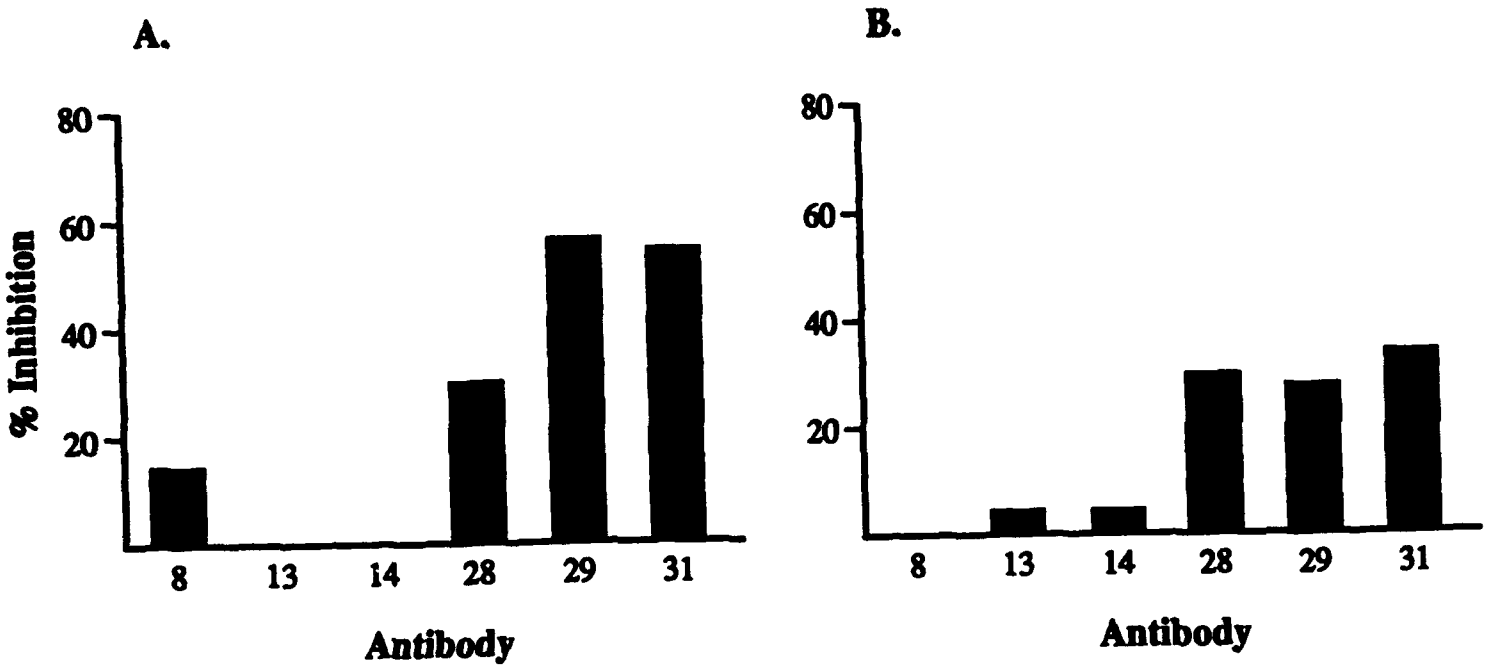


FIGURE 3.

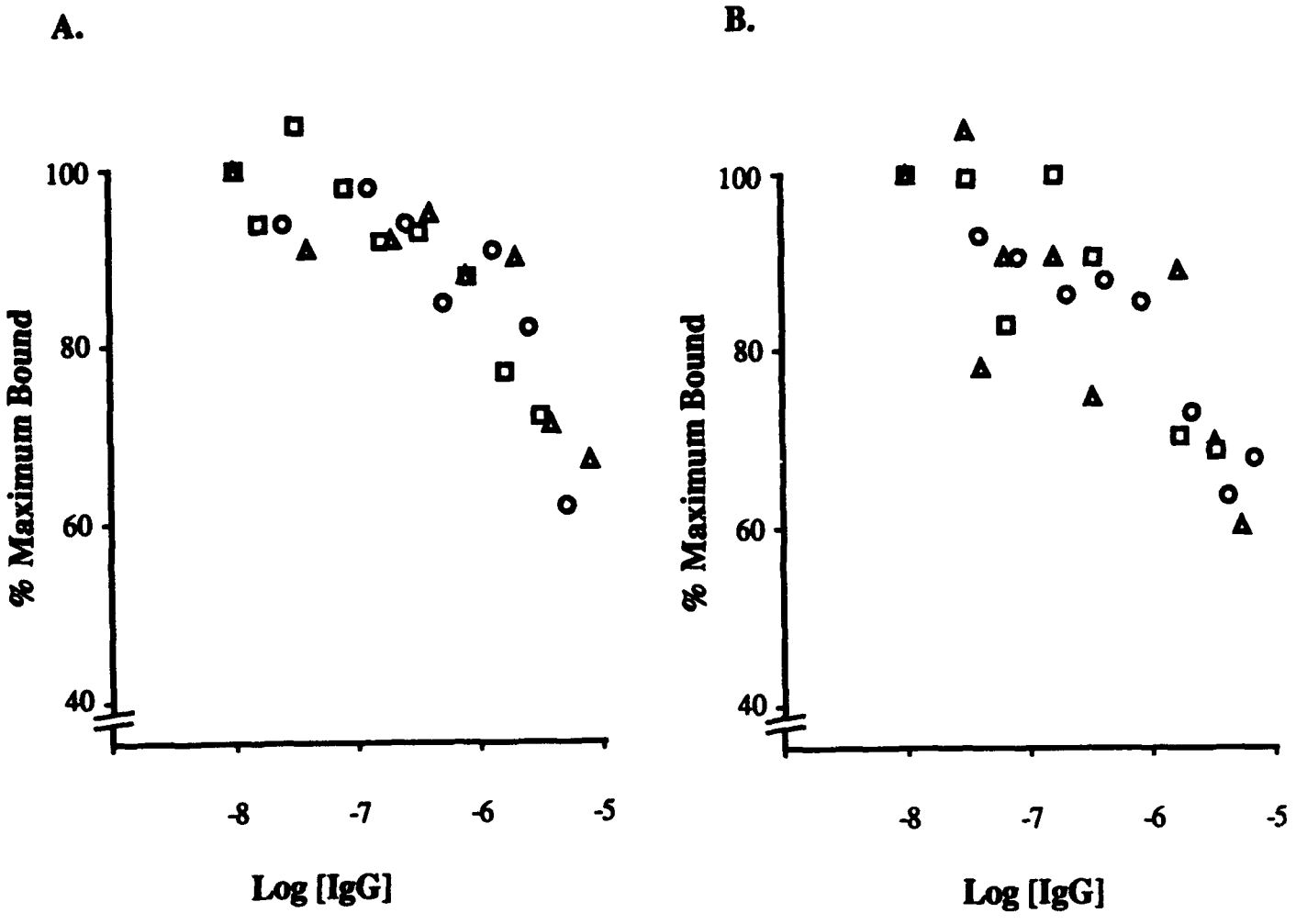
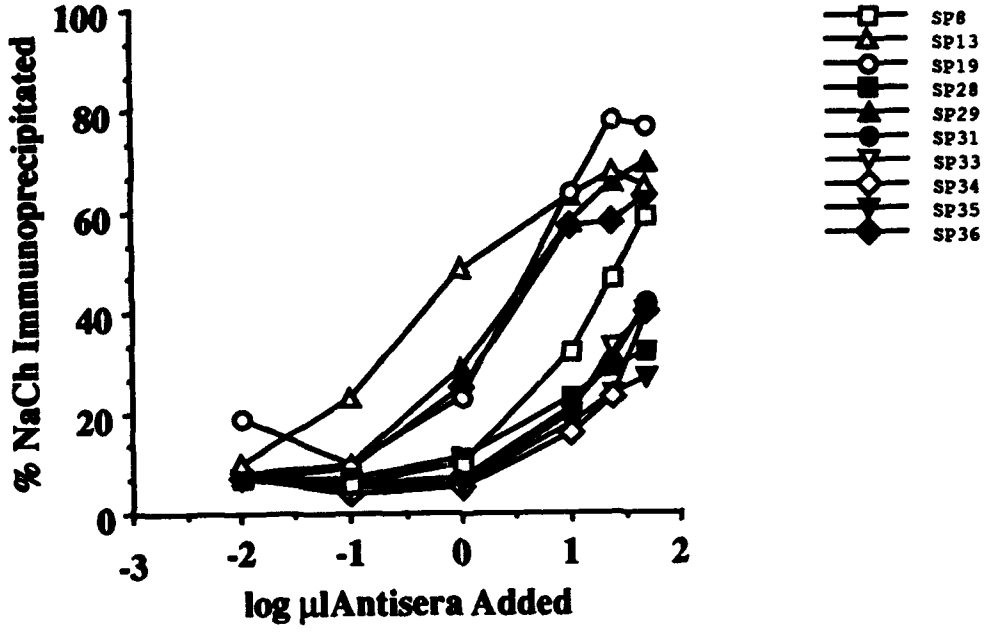


FIGURE 4.

A.


% NaCh Immunoprecipitated
100
80
60
40
20
0
-3
-2
-1
0
1
2
log µlAntisera Added
SP6
SP13
SP19
SP28
SP29
SP31
SP33
SP34
SP35
SP36

B.

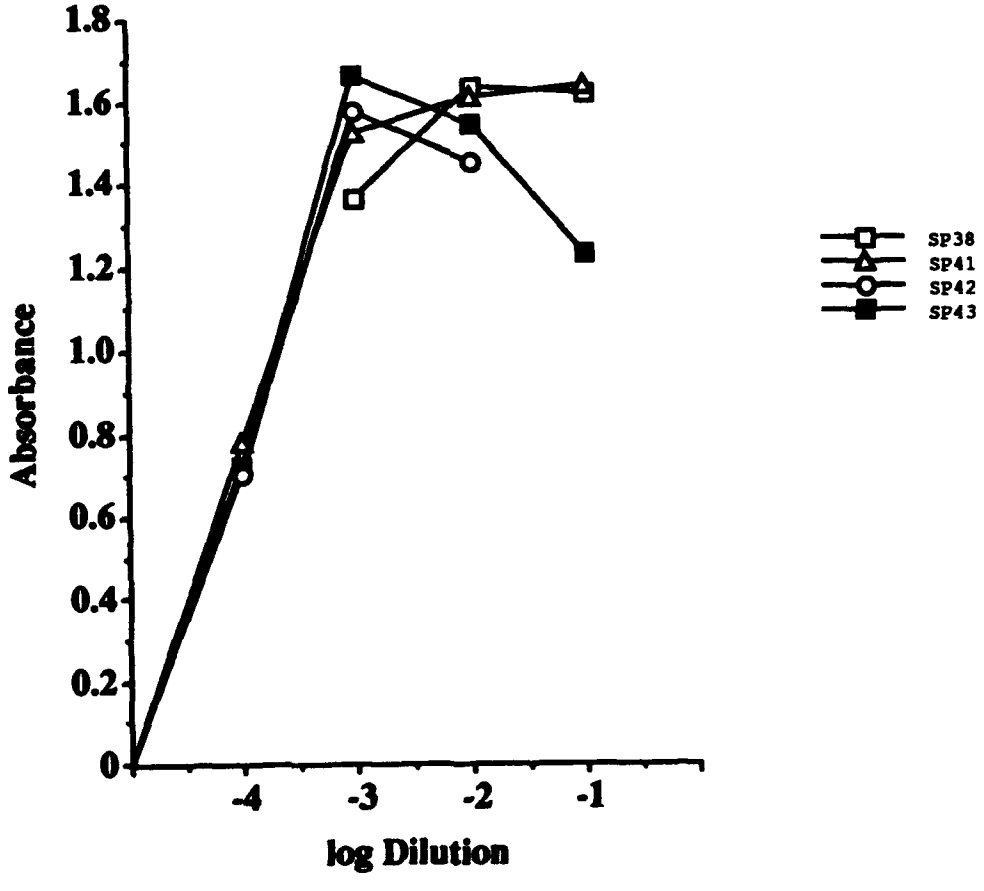

Absorbance
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-4
-3
-2
-1
log Dilution
SP38
SP41
SP42
SP43

FIGURE 5.

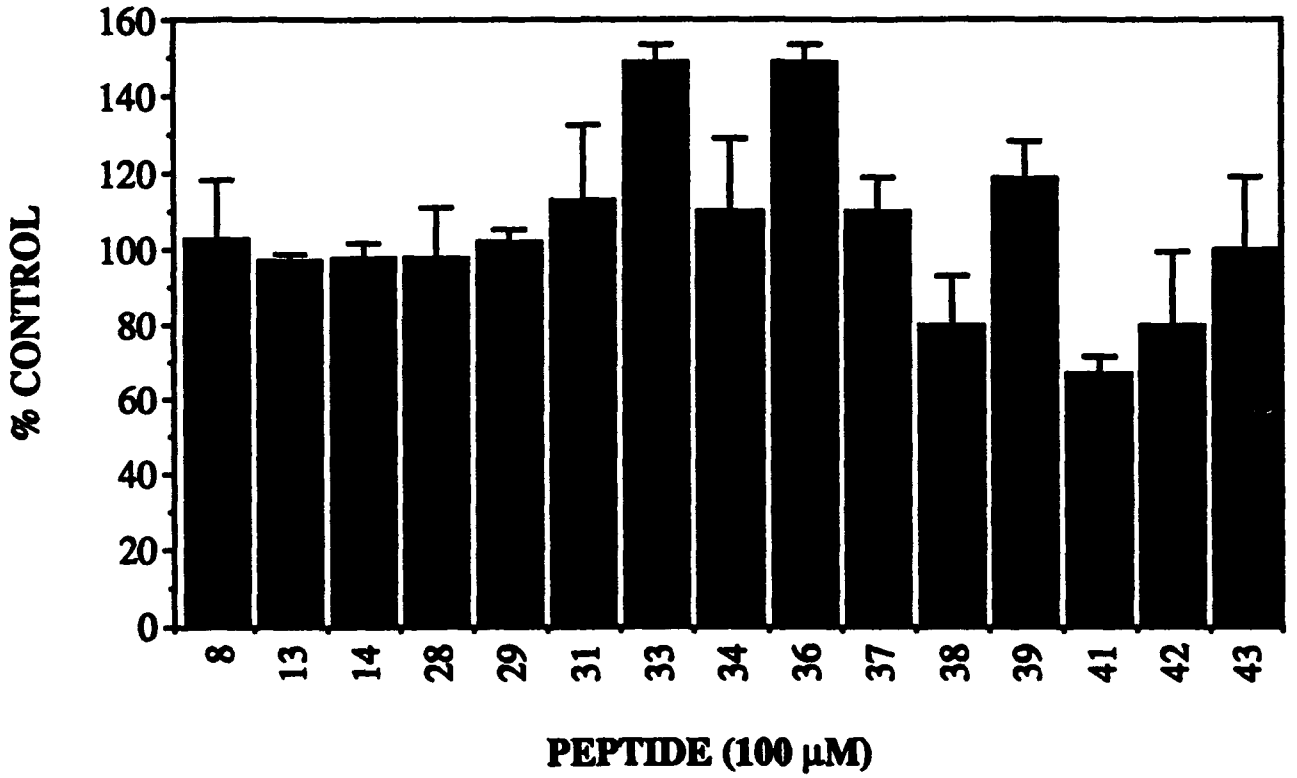
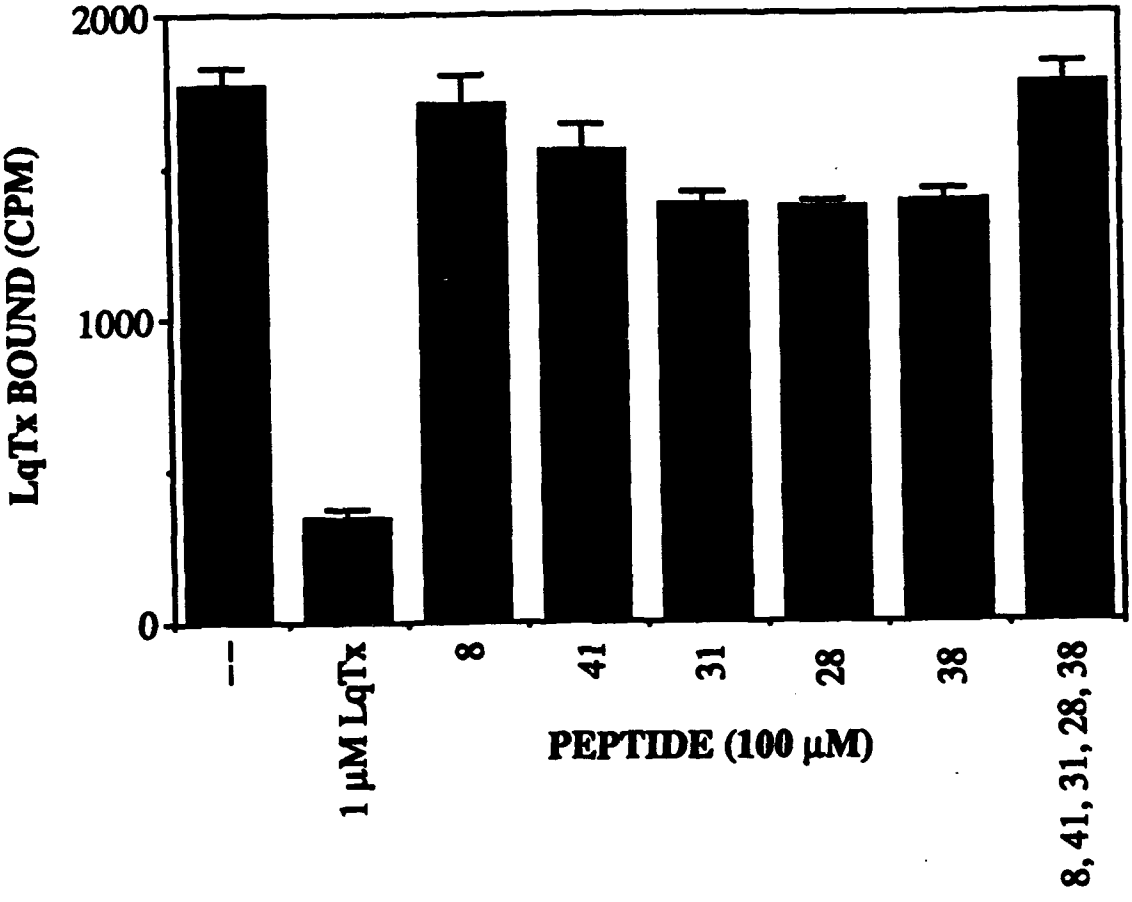

160
140
120
100
80
60
40
20
0
% CONTROL
8
13
14
28
29
31
33
34
36
37
38
39
41
42
43
PEPTIDE (100 μM)

FIGURE 6.


2000
1000
0
LqTx BOUND (CPM)
–
1 μM LqTx
8
41
31
28
38
8, 41, 31, 28, 38
PEPTIDE (100 μM)

TASK II. INHIBITION OF ALPHA SCORPION TOXIN ACTION

A major goal of this contract is to produce antibodies or peptide reagents that prevent the toxic actions of neurotoxins that act on sodium channels. Effective inhibition of the binding of α-scorpion toxins should prevent toxin-induced modification of sodium channel function. As a first step in studying the ability of anti-peptide antibodies to prevent the actions of α-scorpion toxins, we tested the effects of anti-peptide antibodies that reduce α-scorpion toxin binding on the actions of the scorpion toxins on sodium channels in rat brain neurons in primary cell culture and in transfected cells by patch clamp recording in the whole cell voltage clamp configuration.

Experimental Procedures

Cell Culture. Brains from 19 day fetuses were dissociated in a solution of 20 mg/ml trypsin and calcium- and magnesium-free PBS for 40 min at 37°C with shaking every 5 min. The enzymatic reaction was stopped by the addition of 20 ml of DMEM culture medium containing 5% fetal bovine serum and 10% heat-inactivated horse serum. The solution was centrifuged at 1000 RPM for 10 min, the supernatant was aspirated, and the pellet was resuspended in 30 ml of culture medium. Centrifugation and resuspension of the pellet was done three times. On the final resuspension of the pellet, the solution was passed through a nitex filter and cells were plated into 35 mm tissue culture dishes containing sterile poly-d-lysine coated glass coverslips at a density of $1\text{-}3\text{x}10^6$ cells/plate. Cells were maintained in culture for 2 weeks and feed every three days with culture medium. Electrophysiological recording of cells adhering to the coverslips was started three days post plating.

CNaIIA-1 cells were maintained in a culture in RPMI media supplemented with 10% fetal bovine serum as described by West et al (1992).

Antibody Purification. Anti-SP28, anti-SP29, and anti-SP31 antibodies, which inhibit α-scorpion toxin binding, were examined for specific inhibition of scorpion toxin action and anti-SP19, anti-SP20 and nonimmune IgG were used as controls. A Protein-A-Sepharose column equilibrated in 0.1M NaH_2PO_4, pH 8.1 was used to purify the anti-peptide antibodies. A binding buffer (0.15 M NaCl, 0.02 M Tris, pH 8.1) was added to the antisera (1:1 ratio) and shaken for 5 min at 4°C. The antiserum was then added to the Protein-A column followed by an 5-fold excess of binding buffer. Fractions of 25 drops were collected and A_{280} for each was read. When the absorbance returned to zero, indicating the column was washed free of residual proteins, the bound antibodies were eluted from the column using 0.1M glycine, pH 3. Twenty-five drop fractions were collected, fractions with A_{280} greater than 1.0 were pooled, and the pH was adjusted to neutrality. The pooled fraction of antibodies was then dialyzed into external electrophysiological recording solution by repetitive concentration and dilution by centrifuging at 5000 RPM for 1 hr in a Centricon 10 or by overnight dialysis using the constant perfusion pump connected to a microdialyzer.

Electrophysiological Recordings. Brain cells were recorded in whole cell voltage clamp mode (Hamill, 1981). A reverse sodium gradient was used in the brain cells to prevent regenerative action potentials from distal processes from invading the soma. The internal solution was (in mM) 115 Choline-Cl, 10 NaCl, 20 TEA-Cl, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5 HEPES, 5 dextrose, pH 7.4. The external recording solution contained contained (in mM) 150 NaCl, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 5 dextrose, pH 7.4. Coverslips containing cells were placed in a recording chamber 200 µL in volume. For each cell, control records were taken that include both activation and inactivation paramaters. A 10 µL volume of antibody solution (0.6-2 µM final concentration) was then added to the recording chamber and effects were examined for a 10 min period. Following this period, 10 µl of α-LqTx was added to the chamber and its effects were examined for 10 min.

Normal sodium gradients were used in the whole-cell voltage clamp mode (Hamill et al, 1981). The internal recording solution is (in mM) 90 CsF, 60 CsCl, 10 NaCl, 5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES, 5 dextrose, pH 7.3. Coverslips were incubated in 10 μM antibody solution. Cells were recorded in the antibody solution for approximately 10 min. Ten μl of α-Lqtx (6-100 nM) in recording solution plus bovine serum albumin (24.2 μM) were added and allowed to diffuse throughout the 200 μL chamber. The effect of α-LqTx on sodium channel inactivation was then recorded.

Results

Our experiments have focussed primarily on anti-SP31 as a test antibody, but anti-SP28 and anti-SP29 were also studied. In experiments developing methods for antibody and toxin application, 31 rat brain neurons were successfully voltage clamped, and 12 were controlled long enough for both the antibody and the α-Lqtx effects to be examined. Antibody concentrations ranged from 0.4 μM to 2 μM. The concentration of α-LqTx was 100 nM; this concentration had a maximal effect on slowing sodium channel inactivation on all cells tested. However, the effect of the antibodies differed from cell to cell. In some cells, pre-incubation with anti-SP31 effectively prevented the effects of α-Lqtx on the time course of sodium channel inactivation (Fig 1). In other cases, α-Lqtx slowing of inactivation was not measurably affected by pre-exposing the cells to anti-SP31 (Fig. 1). These results suggested variability in antibody access or antibody binding to individual brain neurons.

In order to reduce the variability that may arise from use of a mixed population of rat brain neurons as a test cell type, we have taken advantage of the development of CNaIIA-1 cells (West et al, 1992), which express transfected Type IIA sodium channels, to continue the analysis of the effects of anti-peptide antibodies on α-scorpion toxin action in the context of a cloned sodium channel expressed in an essentially electrically silent clonal cellular background. Anti-SP31 was analyzed at a concentration of 10 μM in recording solutions and cells were incubated in this solution at room temperature or 37°C for at least 30 min before Lqtx was applied. The toxin concentration was 6 nM, and the solution contained 24.2 μM BSA. Ninety one CNaIIA-1 cells were successfully voltage-clamped, and 51 were controlled long enough to examine the effects of antibodies and α-Lqtx. The results varied from cell to cell as for brain neurons. In some cells, α-Lqtx added after pre-incubation with anti-SP31 did not change the time course of inactivation (Fig. 2) suggesting substantial block of toxin binding. In other cells, the effect of α-Lqtx on slowing of inactivation was not affected by pre-exposing the cells to anti-SP31 (Fig. 2). Twenty cells were studied with anti-SP19, anti-SP20, and nonimmune IgG without evidence of inhibition of α-LqTx action.

Similar results were obtained with experiments using anti-SP28 and anti-SP29 in a smaller number of cells. As described above, the effects of scorpion toxin on some cells was reduced by the antibodies while others showed little response. The onset of toxin effect was slowed more than the steady state toxin effect was reduced suggesting that access of the toxin to its receptor site is impeded. .

Discussion

Overall, the electrophysiological experiments to examine functional block of α-scorpion toxin action indicate that only partial inhibition can be obtained with anti-SP28, anti-SP29, and anti-SP31. These results are consistent with the partial inhibition of α-LqTx binding observed in the experiments described under Task I, even at apparently saturating antibody concentrations. Evidently, a substantial fraction of the sodium channels in these cells are inaccessible to antibody binding and therefore are resistant to antibody block of toxin action. The segments of the sodium channel α subunit that have implicated in scorpion toxin binding are on extracellular loops near clusters of potential N-linked glycosylation sites. Carbohydrate chains on membrane glycoproteins

can be variable in structure and conformation. The extensive carbohydrate chains on these regions of the α subunit may be able to prevent antibody binding to a variable degree depending on their structure or conformation on individual sodium channels or in individual cells. Future experiments with our new series of anti-peptide antibodies may resolve this problem by identifying a site for antibody targeting that is consistently accessible for antibody binding.

Figures

FIG. 1. Selected current recordings of the effects of anti-SP31 on α-LqTx action in rat brain neurons. Whole cell sodium currents were recorded using reverse sodium gradients (see Experimental Procedures). The cells were held at -80 mV and depolarized to a test pulse potential of 0 mV to elicit outward sodium currents. **A.** In a cell pre-exposed to anti-SP31, the antibody blocked the α-LqTx effect on the sodium current. Current traces recorded 0, 4, and 10 min after exposure to 100 nM α-LqTx. **B.** A different cell examined as in panel A demonstrating a lack of effect of anti-SP31 on α-LqTx effects. Current traces were recorded 0 or 2 min after exposure to 100 nM α-Lqtx. **C.** A control cell that was not exposed to anti-SP31. The current traces were recorded after 0 and 2 min exposure to 100 nM α-Lqtx.

FIG. 2. Selected current recordings of the effects of anti-SP31 on α-LqTx action in CNaIIA-1 cells. Whole cell sodium currents were recorded using normal sodium gradients (see Experimental Procedures). The cells were held at -80 mV and depolarized to a test pulse potential of 0 mV to elicit inward sodium currents. **A.** In a cell pre-exposured to anti-SP31, the antibody blocked the effect of α-LqTx on the sodium current. Current traces were recorded 0 and 5 min after exposure to α-Lqtx. **B.** A different cell examined as in panel A demonstrating a lack of effect of pre-exposure to anti-SP31 on α-LqTx effects. Current traces were recorded after 0 and 5 min exposure to 6 nM α-LqTx. **C.** A control cell that was not exposed to anti-SP31. Current traces were recorded 0 and 1.25 min after exposure to 6 nM α-Lqtx.

FIGURE 1.

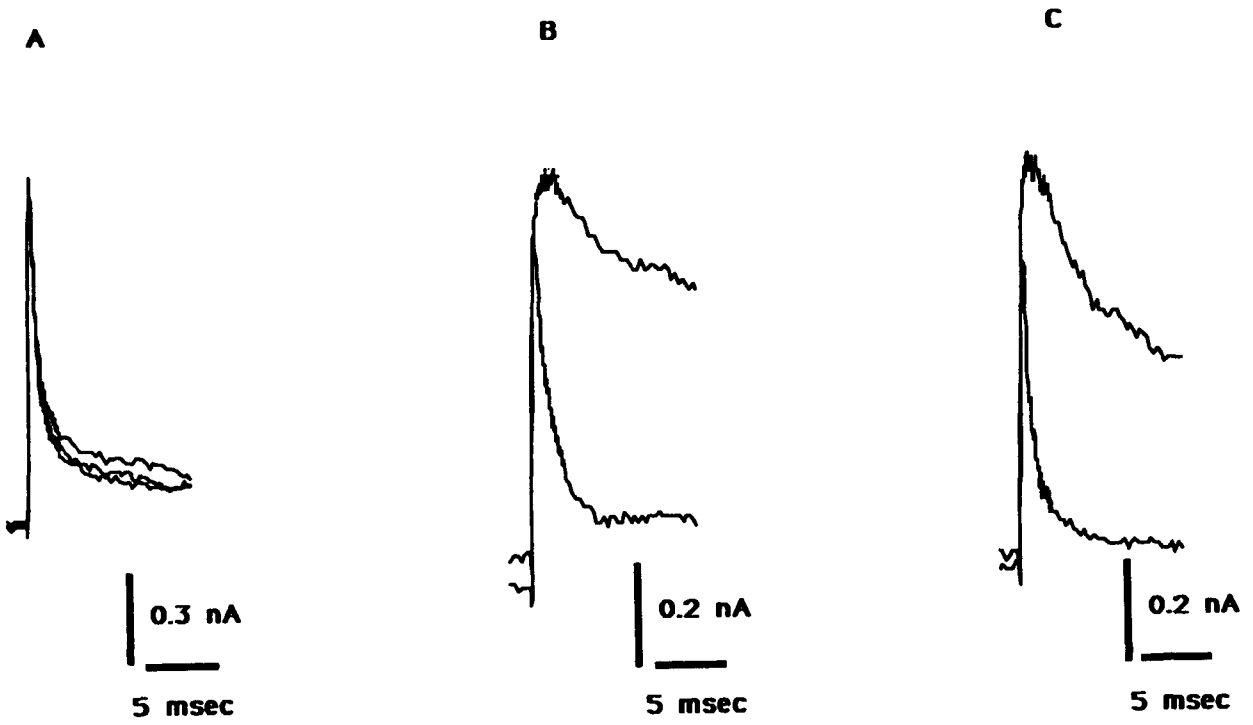
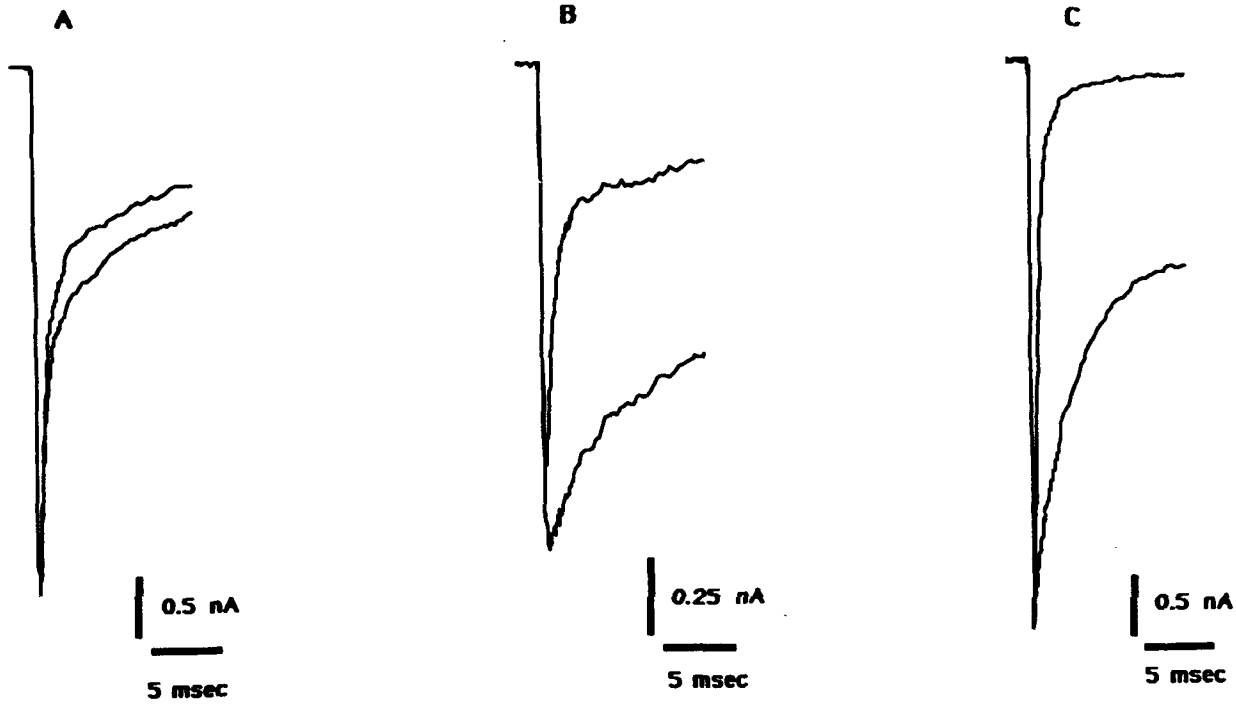


FIGURE 2.



TASK III. COVALENT LABELING OF NEUROTOXIN RECEPTOR SITES 1 AND 5

Experiments described under this Task are directed toward covalent labeling of neurotoxin receptor site 1 using photoreactive derivatives of tetrodotoxin and neurotoxin receptor site 5 using photoreactive derivatives of brevetoxins. So far, we have carried out some preliminary tests with derivatives of tetrodotoxin to label neurotoxin receptor site 1 and we have focused more intensively on photolabeling of neurotoxin receptor site 5 with photoreactive derivatives of *Ptychodiscus brevis* toxin 3 (PbTx3). These experiments were carried out in collaboration with the laboratory of Dr. Daniel Baden, University of Miami.

Neurotoxin receptor site 1

The low specific activity of ^{3}H labeled compounds slows the progress of antibody mapping experiments because it requires larger amounts of sodium channel protein for analysis and radiolysis degrades the photoreactive group requiring frequent re-synthesis of the labeled probe. Preliminary experiments were carried out to test the efficacy of a biotinyl derivative of tetrodotoxin as a covalent label for neurotoxin receptor site 1. The nonradioactive biotin label is stable and can be detected with high sensitivity by high affinity binding of an ^{125}I-labeled derivative of avidin, a biotin-binding protein with a K_d of 10^{-15} M. This approach has potential advantages in providing a more sensitive and stable probe of neurotoxin receptor than offered by present derivatives of tetrodotoxin. The biotinyl tetrodotoxin derivative tested had an apparent K_d for the rat brain sodium channel of 230 nM compared to 20 nM for tetrodotoxin at this sodium channel. This K_d is higher than desirable for efficient photoaffinity labeling. Initial experiments to detect tetrodotoxin incorporation into the α subunit by blot overlay methods were unsuccessful.

Neurotoxin receptor site 5

Experimental Procedures

Materials. Sephacryl S-300 was obtained from Pharmacia and Soluene 350 tissue solubilizer from Packard Instrument Co. All electrophoresis chemicals and 2-mercaptoethanol were purchased from Fisher Scientific Co. Organic counting scintillant (OCS), standard high molecular weight rainbow markers, and sodium borotritiide were from Amersham Corp. Aquasol was purchased from New England Nuclear. Neuraminidase type X from *Clostridium perfringens*, BSA, phosphatidylcholine type III-E from egg yolk, and all protease inhibitors were from Sigma Chemical Corp. All remaining chemicals were reagent grade or better and were purchased from Sigma. All enzymes were used without further purification.

Toxin purification. Brevetoxins PbTx2 and PbTx3 were extracted from stationary phase laboratory cultures of *Pychodiscus brevis* by a combination of chloroform/methanol extraction, thin layer chromatography and reverse phase HPLC (Baden et al., 1981; Poli et al., 1986). Both [^{3}H]PbTx3 and unlabeled PbTx3 were synthesized by reduction of the PbTx2 aldehyde using sodium borotritiide or sodium borohydride respectively (Poli et al., 1986). Specific activity of the radioactive preparation was determined by HPLC mass quantification at 215 nm and scintillation spectroscopy against [^{3}H]methanol standards. Tritiated PbTx3 used for synthesis of the photoaffinity probe had a specific radioactivity in the range of 10-13 Ci/mmol.

Preparation of synaptosomes. A modification of the method described by Dodd et al. (1981) was used. The brains of Sprague-Dawley rats were removed and homogenized with 10 strokes of a Potter-Elvehjem tissue grinder in homogenization buffer containing 0.32 M sucrose, 5 mM Na_2HPO_4 (pH adjusted to 7.4 with phosphoric acid), and 4 protease inhibitors (0.1 mM

phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 mM 1,10-phenanthroline and 1 μM pepstatin A). The sample was sedimented at 1000 x g for 10 min in a Sorvall RC-5B superspeed centrifuge using an SS-34 fixed angle rotor. The pellet was then re-homogenized in homogenization buffer and centrifuged as before. The 2 supernatant solutions were combined, layered upon 1.2 M sucrose and centrifuged at 105,000 x g in a Beckman L7-55 ultracentrifuge using a SW41 Ti swinging bucket rotor. The interface between the 0.32 M and 1.2 M sucrose layers was aspirated and layered upon 0.8 M sucrose. Samples were sedimented at 115,000 x g for 25 min in the ultracentrifuge using the same rotor as before. The pellet contained synaptosomes which were used in binding experiments at a concentration of approximately 0.1 mg/ml. This preparation was found to retain activity for several months when stored at -70° C.

Synthesis of p-azido benzoic acid. A modification of the method described by Rao and Venkataraman (1939) was used. Ten mg of 4-aminobenzoic acid was reacted with a solution of 25% HCl (50 μl) and diazotized with a solution of sodium nitrite (4.5 mg in 13 μl H_2O) at 0° C. Excess urea was added after 20 min to destroy the remaining nitrous acid and a solution of sodium azide (4.6 mg in 13 μl H_2O) was added dropwise. The precipitated triazobenzoic acid was incubated 1 hr on ice. The solvent was flash-evaporated and the reaction product stored in the dark.

Synthesis of p-azido benzoate-linked brevetoxin. Synthesis was carried out in the dark or under red photography lights. Carbonyldiimidazole (0.162 g) was reacted with 0.162 g p-azidobenzoic acid by addition of dry benzene to give a final volume of 10 ml. One mg of [^{3}H]PbTx3 was dried under nitrogen, then desiccated for 10 min over P_2O_5 using high vacuum. One ml of reacted p-azidobenzoic acid was added to the toxin, sealed in a thick walled glass vial, and heated in a mineral oil bath for 24 hr at 70° C. The reaction product was dried under nitrogen, resuspended in acetone and plated on a 500 μm silica gel plate in petroleum ether:acetone (70:30). The desired fraction was visualized by exposing a small portion of the plate to UV light. The photoaffinity-labeled brevetoxin was scraped from the plate and eluted in acetone. The solvent was flash-evaporated and the reaction product purified by HPLC at 215 nm.

Reversible binding experiments. Binding of [^{3}H]PbTx3 p-azido benzoate (hereafter designated as [^{3}H]PbTx3-Pho) was determined using a rapid centrifugation technique in binding medium containing 50 mM HEPES (adjusted to pH 7.4 with Tris base), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/ml BSA, 0.01% (w/v) Emulphor EL-620 (a nonionic emulsifier, GAF Corp.). Synaptosomes, at a final protein concentration of 100 μg/ml, were added to reaction vials containing [^{3}H]PbTx3-Pho (total binding). For Scatchard analyses, toxin concentrations ranged from 0 to 100 nM. Nonspecific binding was determined in the presence of a saturating concentration of unlabeled [^{3}H]PbTx3-Pho (10 μM). After mixing and incubating for one hour at 0° C in the dark, samples were centrifuged for 2.5 min at 15,000 x g. Supernatant solutions were sampled for free [^{3}H]PbTx3-Pho and pellets were washed with three drops of ice cold wash medium containing 5 mM HEPES (pH 7.4), 163 mM choline chloride and 1 mg/ml BSA. Pellets were transferred to counting vials and bound radioactivity determined by scintillation spectroscopy. The entire experiment was carried out in subdued room light or under red photography lamps. Specific binding was determined by calculating the difference between total and nonspecific binding (Poli et al., 1986). The results are presented as mean and standard error; where error bars are not visible, the errors were smaller than the symbols.

Photoaffinity labeling. Synaptosomes in Petri dishes were irradiated (18 W, at a distance of 1 cm) with UV light at a wavelength of 254 nm for 5 min at 0°C.

SDS-PAGE. Photoaffinity labeled samples were centrifuged for 2.5 min at 15,000 x g and pellets were prepared for SDS-PAGE by resuspension in non-reducing sample buffer (Laemmli, 1970) and incubation at 100° C for 2 min. Samples were loaded on 1.5 mm thick SDS acrylamide gels (either 5-15% or 5-20% gradients or single polyacrylamide concentration). Electrophoresis was performed at 75 mV through the stacking gel and at 200 mV through the resolving gel. Gels were stained with 2% Coomassie brilliant blue in methanol:acetic acid:H_2O

(50:7.5:42.5) for one hour, then destained in methanol:acetic acid:H_2O (30:7.5:62.5) to locate protein bands (Weber and Osborn, 1969).

Gel slicing. Photolabeling and SDS-PAGE were carried out as described above. Gels were left unstained prior to slicing due to the quenching properties of Coomassie dye. They were sliced into 4 mm thick pieces with a razor blade and approximate molecular weights were recorded by comparison to molecular weight markers in adjacent lanes. Slices were dissolved in 0.5 ml Soluene and 0.1 ml H_2O by heating at 60° C for 3 hours. Fifty microliters glacial acetic acid was added to neutralize the scintillation cocktail (OCS). After adding 6 ml OCS, samples were placed in the dark overnight, then assayed for radioactivity.

Purification and reconstitution of sodium channels. For reconstitution experiments, sodium channels purified from rat brain by solubilization in Triton X-100, chromatography on DEAE-Sephadex, hydroxylapatite, and wheat germ agglutinin-Sepharose, and sedimentation through sucrose gradients as described by Hartshorne and Catterall (1984) were reconstituted into phosphatidylcholine/phosphatidylethanolamine vesicles as described by Feller et al (1985).

Results

Covalent Labeling of Sodium Channels in Synaptosomes by a Photoreactive Brevetoxin Derivative

Receptor stability to irradiation. It was necessary to determine whether the Na^+ channel receptor was destroyed by UV light used to activate the photoaffinity label. Synaptosomes were irradiated as described above and then incubated with 2 nM [^{3}H]PbTx3-Pho for 1 hr at 4° C. Bound radioactivity was assayed for determination of total and nonspecific binding. Approximately 11% of synaptosome specific binding capacity was lost upon irradiation in comparison to a control population which had not been exposed to UV light.

Displacement of native [^{3}H]PbTx3 by photoaffinity probe. In order for the photoaffinity derivative of PbTx3 to be used in specific labeling of the brevetoxin binding site 5, the derivatized toxin must demonstrate the ability to displace underivatized toxin from its receptor. To examine this, specific binding of increasing concentrations [^{3}H]PbTx3 was measured in the absence of competing PbTx-Pho or in the presence of 5 nM or 15 nM PbTx-Pho and the data were analyzed for competitive inhibition of binding by double reciprocal plots. The photoreactive compound displaced [^{3}H]PbTx3 from its specific site of binding in a competitive manner, as evidenced by the intersection of the double reciprocal plots on the y-axis (Fig. 1). The double reciprocal analysis indicates a K_d for PbTx-Pho of 5 nM. The similar binding affinities of derivatized and native PbTx3 illustrate that accessibility of the brevetoxin to its receptor site is not limited by covalent modification of the toxin hydroxyl function by the azido group of the photoreactive compound.

Specific binding characteristics of [^{3}H]PbTx-Pho. Analysis of the time necessary to remove one half the total [^{3}H]PbTx3-Pho from its receptor ($t_{1/2}$) showed that the $t_{1/2}$ of the photoaffinity probe was approximately 2 hr, a value significantly higher than the 20-30 min $t_{1/2}$ calculated for native PbTx3. This long half-life of the toxin-receptor complex allowed extensive washing to remove nonspecifically bound toxin before irradiation. The most effective blocker of nonspecific binding was the nonradioactive PbTx3-Pho derivative which displaced about 59% of the total bound [^{3}H]PbTx3-Pho consistent with 59% specific binding. The specific binding values of these preparations were improved by use of bovine serum albumin (BSA) as a scavenger protein. Binding medium containing 1% BSA reduced nonspecific binding substantially, resulting in approximately 85% specific binding (Table I). Three washes by dilution and centrifugation prior to photolysis resulted in a decrease in nonspecific binding without significant reduction in specific binding. All photoaffinity labeling experiments with the photoaffinity label utilized three pre-UV washed with BSA and a 5 minute irradiation time to maximize total binding while minimizing nonspecific interaction.

Scatchard analysis. A Scatchard analysis of photoaffinity probe binding to synaptosomal membranes resulted in a non-linear plot, suggesting the presence of two binding sites for the brevetoxin molecule on the Na^+ channel (Fig. 2). Binding of the photoaffinity label to the high affinity site was described by a Kd of 0.64 nM and a maximal binding affinity (Bmax) of 3.74 pmol/mg. Two site binding of [^{3}H]PbTx3-Pho to synaptosomal membranes was substantiated in an experiment which utilized toxin concentrations up to 100 nM (Fig. 3A). High affinity site Kd was 1.04 nM and Bmax = 6.28 pmol/mg whereas low affinity site values were 15.6 nM and 39.6 pmole/mg for Kd and Bmax, respectively.

High affinity binding can be blocked by incubating synaptosomes with 5 nM unlabeled PbTx3-Pho followed by irradiation to form a covalent bond between ligand and receptor. A Scatchard analysis of this pre-treated preparation was compared to a control experiment (Fig. 3B) using an identical concentration of synaptosomes which had not been pretreated. Pre-treated synaptosomes displayed only low affinity binding.

Analysis of photolabeled sodium channels by SDS-PAGE. SDS-PAGE analysis of [^{3}H]PbTx3-Pho covalently bound to synaptosomes was performed using both 5-17.5% and 5-20% gradient gels as well as 5% acrylamide gels. The gradient gels were used to resolve potential binding of the photoaffinity probe to both the $\alpha\beta 2$ subunit complex (about 300 kD) and the $\beta 1$ subunit (36 kD) on the same gel. A protein band with a molecular weight slightly greater than the 200 kD marker protein was determined to contain the most specifically bound radioactivity. Since this band was located in such close proximity to the stacking gel which also contained radiolabelled proteins unable to enter the resolving gel, a 5% gel was used to separate these two components and to provide direct evidence of labeling of $\alpha\beta 2$ with the radiolabeled photoaffinity probe (Fig. 4). This photoaffinity labeled α subunit preparation was used for antibody mapping of the site of covalent labeling as described below under Task V.

Covalent Labeling of Sodium Channels in Reconstituted Vesicles by a Photoreactive Brevetoxin Derivative

High affinity binding of PbTx3 to purified and reconstituted sodium channels. Although sodium channels in synaptosomes can be specifically photolabeled by the photoreactive brevetoxin derivative [^{3}H]PbTx3-Pho, the low concentration of sodium channels in the synaptosomes and the losses that inevitably occur in the purification of the labeled sodium channels from the synaptosomes limit the concentration of purified, photolabeled sodium channels that are available for antibody mapping. This has greatly slowed the progress of the antibody mapping experiments described under Task IV below. We have therefore developed methods to measure the specific high affinity binding of PbTx3 to purified sodium channels in reconstituted phospholipid vesicles. Reconstituted sodium channels were prepared as described under Experimental Procedures. Vesicles were incubated with [^{3}H]PbTx3 as described above and the reconstituted vesicles with bound toxin were collected and washed by filtration on GF/F filters. In the presence of 0.5 nM [^{3}H]PbTx3, reconstituted sodium channels rapidly bind labeled toxin (Fig. 5). Maximum binding is obtained in less than 10 min. The binding of the toxin to reconstituted sodium channels is inhibited up to 94% by unlabeled toxin with a Kd of approximately 10 nM (Fig. 6). Thus, specific binding accounts for 94% of total binding. No specific binding is observed with reconstituted vesicles containing no sodium channels (Fig. 6). PbTx3-Pho competes with [^{3}H]PbTx3 for binding to purified and reconstituted sodium channels. Eadie-Hofstee analysis of the mechanism of inhibition of binding of [^{3}H]PbTx by unlabeled PbTx3-Pho reveals a strictly competitive mode of inhibition (Fig. 7).

Discussion

The results with specific binding and covalent labeling of sodium channels with brevetoxin derivatives are very encouraging. It is surprising that such a hydrophobic ligand binds with such high specificity of sodium channels in both synaptosomes and in purified and reconstituted vesicles. The high level of specific binding observed using these reconstituted vesicles indicates that a substantial improvement in the level of photolabeled sodium channels for antibody mapping can be achieved. Experiments to extend the methods for covalent labeling of synaptosomes to the purified and reconstituted sodium channels for antibody mapping experiments are now in progress.

Figure Legends

FIG. 1. The inhibition of [^{3}H]PbTx3 binding by p-azido benzoyl brevetoxin photoaffinity probe. The photoaffinity derivative was examined for its ability to displace [^{3}H]PbTx3 from its specific site of binding in rat brain synaptosomes using a Lineweaver-Burke double reciprocal analysis. Experiments were carried out as described under Experimental Procedures. The concentrations of [^{3}H]PbTx3 were 5, 10, and 15 nM. Unlabeled photoaffinity probe concentrations were 0 (l), 5 (s), 15 (n) nM. Bound radioactivity (B) was measured as the difference between total and nonspecific values. Free radioactivity (F) was determined by assaying supernatant solutions. Lines are computer-generated first order regressions. Error bars span the range of duplicate determinations.

FIG. 2. Scatchard analysis of [^{3}H]PbTx3-Pho binding to crude synaptosomal preparations. Concentrations of photoaffinity probe ranged from 0 to 25 nM. Concentration of nonradioactive PbTx3-Pho used for determination of nonspecific activity was 10 μM. Error bars span the range of duplicate values and points represent the mean.

FIG. 3. Scatchard analysis of [^{3}H]PbTx3-Pho binding to crude synaptosomal preparations. A. Concentrations of photoaffinity probe ranged from 0 to 100 nM. **B.** Analysis was performed as in panel A but synaptosomes were pretreated for one hr at 0° C with 5 nM nonradioactive PbTx3-Pho. Concentration of nonradioactive PbTx3-Pho used for determination of nonspecific binding was 10 μM. Synaptosomes were irradiated for 5 min with a UV lamp having a 254 wavelength maximum. Error bars span the range of duplicate values and points represent the mean.

FIG. 4. SDS-PAGE analysis of sodium channels in synaptosomes covalently labeled by [^{3}H]PbTx3-Pho on a 5% acrylamide gel. Specific binding was calculated as the difference between total and nonspecific components which were electrophoresed in different lanes. Molecular weight is designated in kDa on the upper abscissa. Gel slices were 4 mm thick. Stacking gel and dye front were not counted.

FIG. 5. Time course for brevetoxin binding to vesicles containing reconstituted sodium channels. Vesicle samples were incubated with 0.5 nM [^{3}H]PbTx3 for the indicated time then assayed for radioactivity by filtration through GF/F filters. Specific binding was determined as the difference between total and nonspecific binding in the absence and presence of 10 μM PbTx3. Points represent mean values and error bars indicate the standard deviation.

FIG. 6. Inhibition of [^{3}H]PbTx3 binding to reconstituted sodium channels by unlabeled PbTx3. Binding of 0.5 nM [^{3}H]PbTx3 to reconstituted sodium channels was determined in the presence of the indicated concentration of unlabeled PbTx3 (circles). Heat inactivated sodium channels (incubated at 37°C for 15 min) were reconstituted into phospholipid vesicles and tested for their ability to bind brevetoxin (triangles). Points represent the mean of duplicate determinations and error bars span the range of values.

FIG. 7. The inhibition of [^{3}H]PbTx3 binding by PbTx3 Pho (2-p-tetrahydropyranoxyl-phenyl, 3-p-azidophenyl propionyl brevetoxin). The iodinatable photoaffinity derivative of brevetoxin was examined for its ability to displace [^{3}H]PbTx3 from its specific site of binding in rat brain synaptosomes by Lineweaver-Burke double reciprocal analysis. The concentrations of [^{3}H]PbTx3 were 10, 1, 0.5, 0.25, 0.12 nM. Unlabeled photoaffinity probe concentrations were 0 (open circles), 0.05 (filled circles), 0.5 (open triangles), 5 (filled triangles) and 50 (open squares). Bound radioactivity (B) was measured as the difference between total and nonspecific values (determined in the absence and presence of 10 μM PbTx3). Free radioactivity (F) was determined by assaying supernatant solutions. Lines are computer-generated first order regressions. Error bars span the range of duplicate determinations.

FIGURE 1.

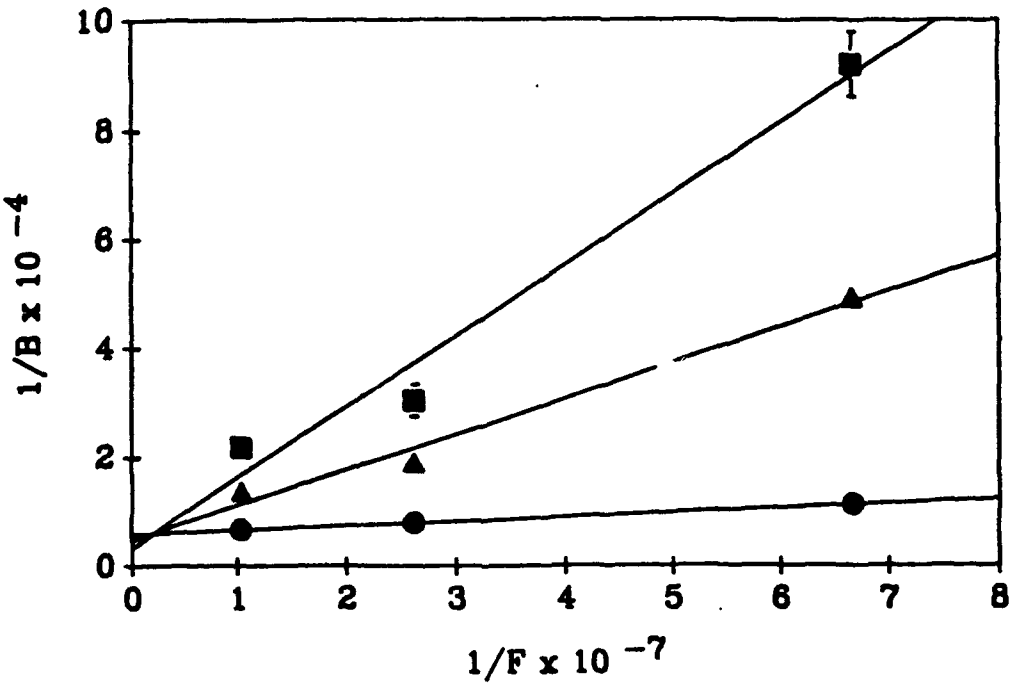


FIGURE 2.

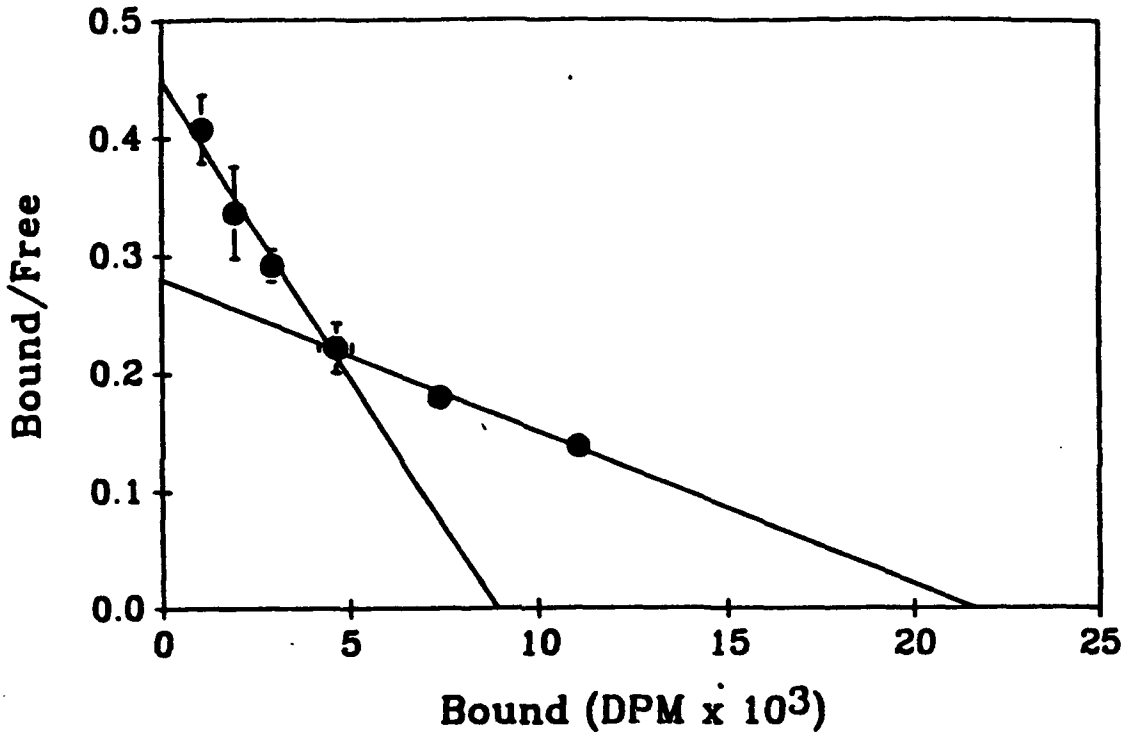


FIGURE 3.

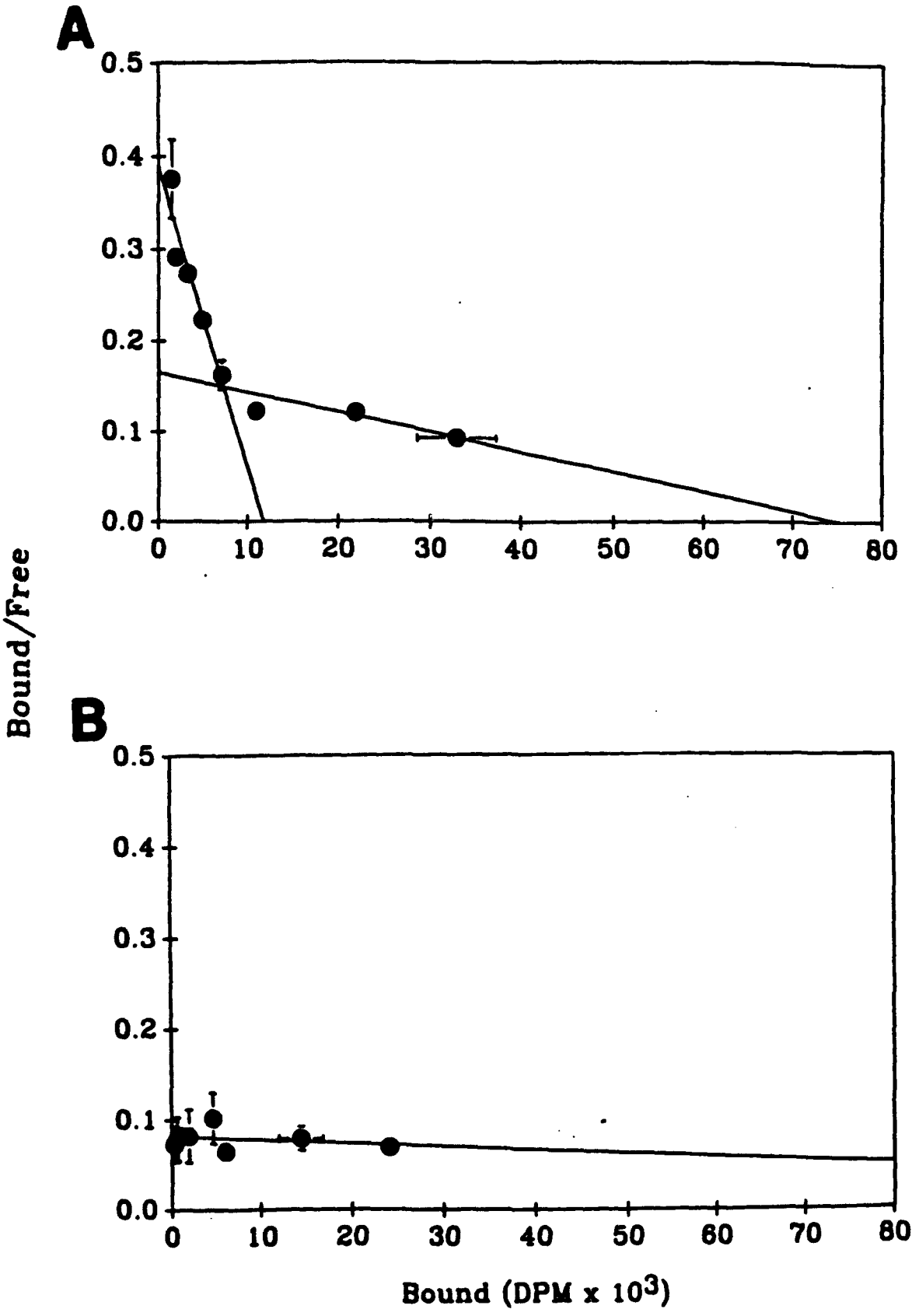

A
B
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
Bound/Free
Bound (DPM x 10^3)

FIGURE 4.

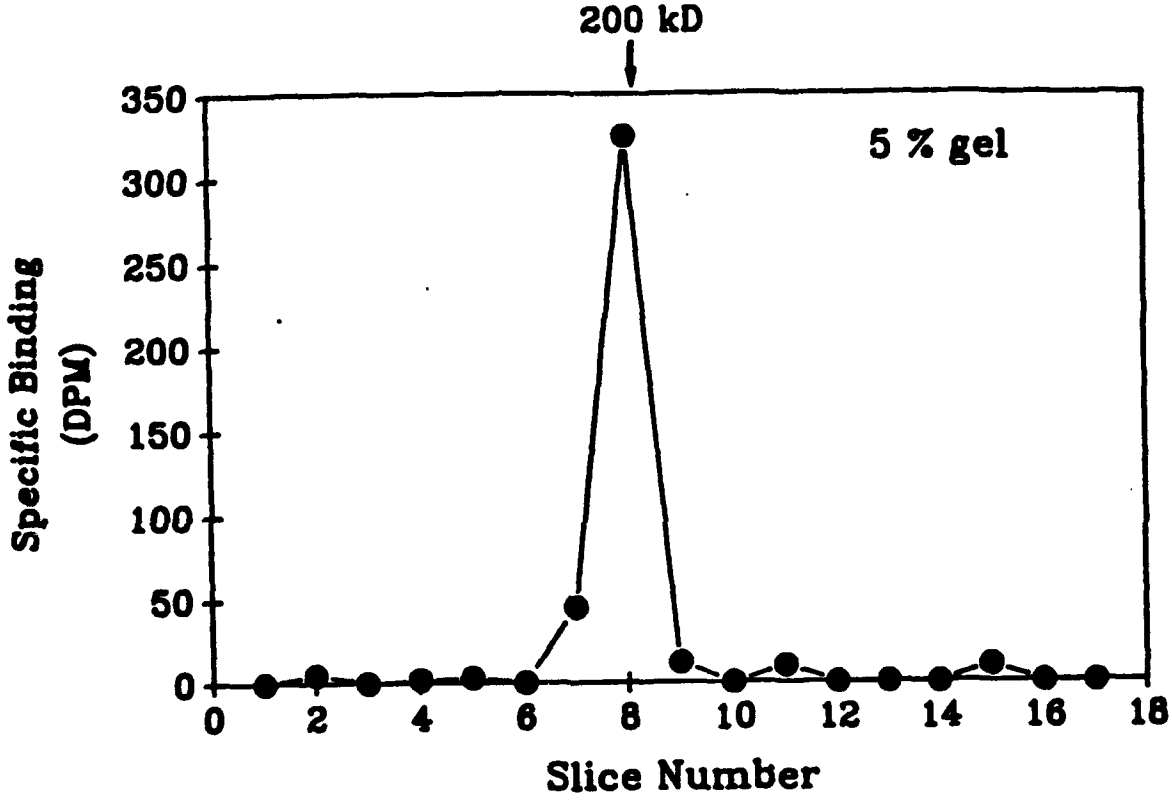

200 kD
5 % gel
Specific Binding
(DPM)
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
18
Slice Number

FIGURE 5.

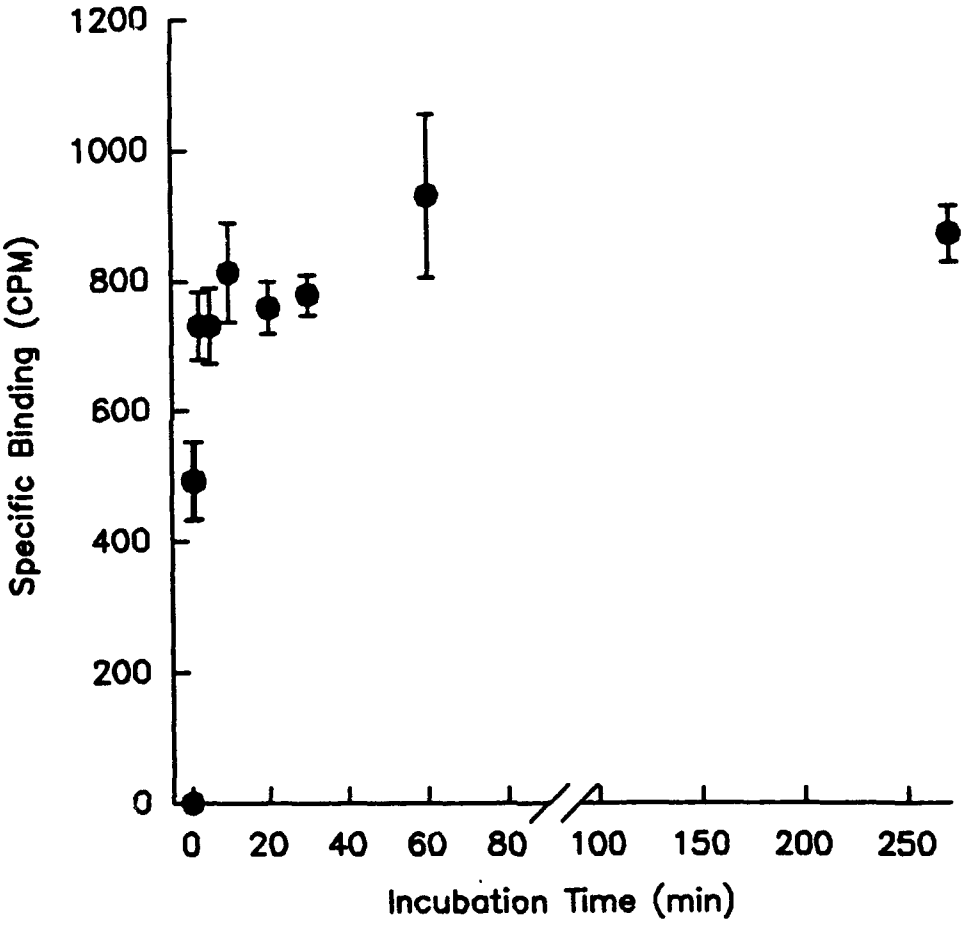

1200
1000
800
600
400
200
0
Specific Binding (CPM)
0
20
40
60
80
100
150
200
250
Incubation Time (min)

FIGURE 6.

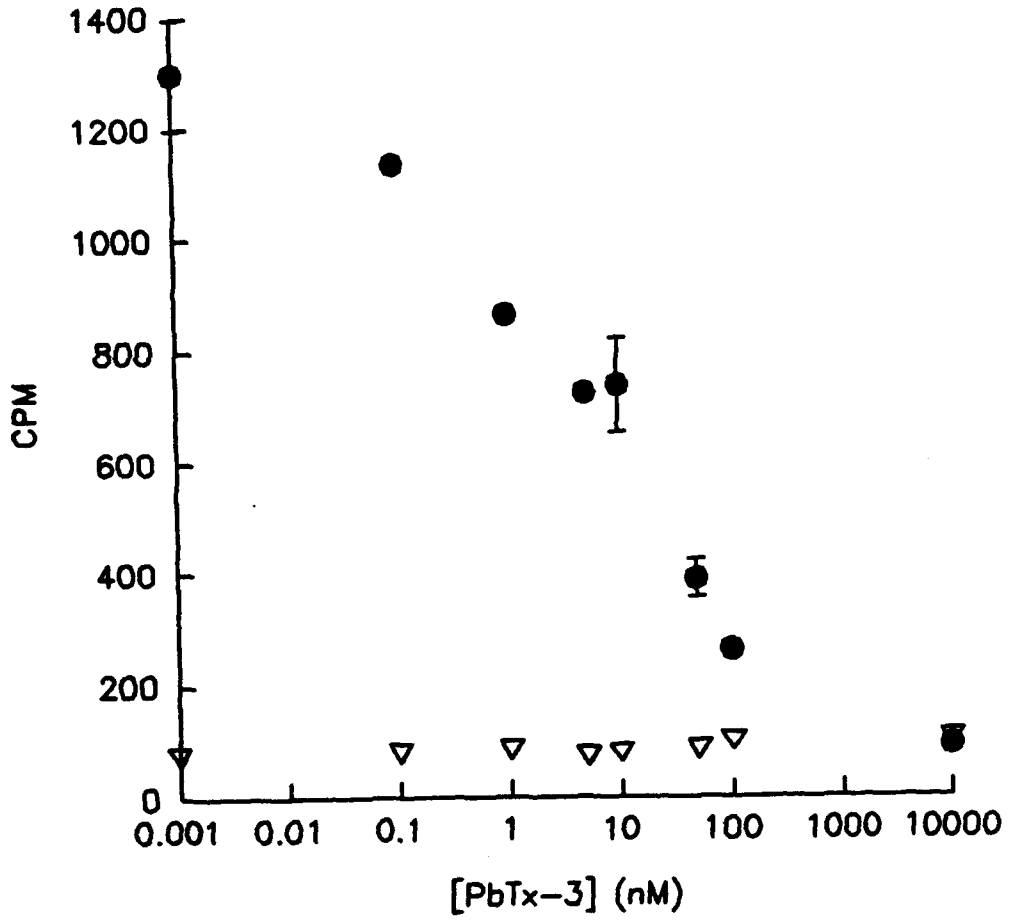

1400
1200
1000
800
600
400
200
0
CPM
0.001
0.01
0.1
1
10
100
1000
10000
[PbTx–3] (nM)

FIGURE 7.

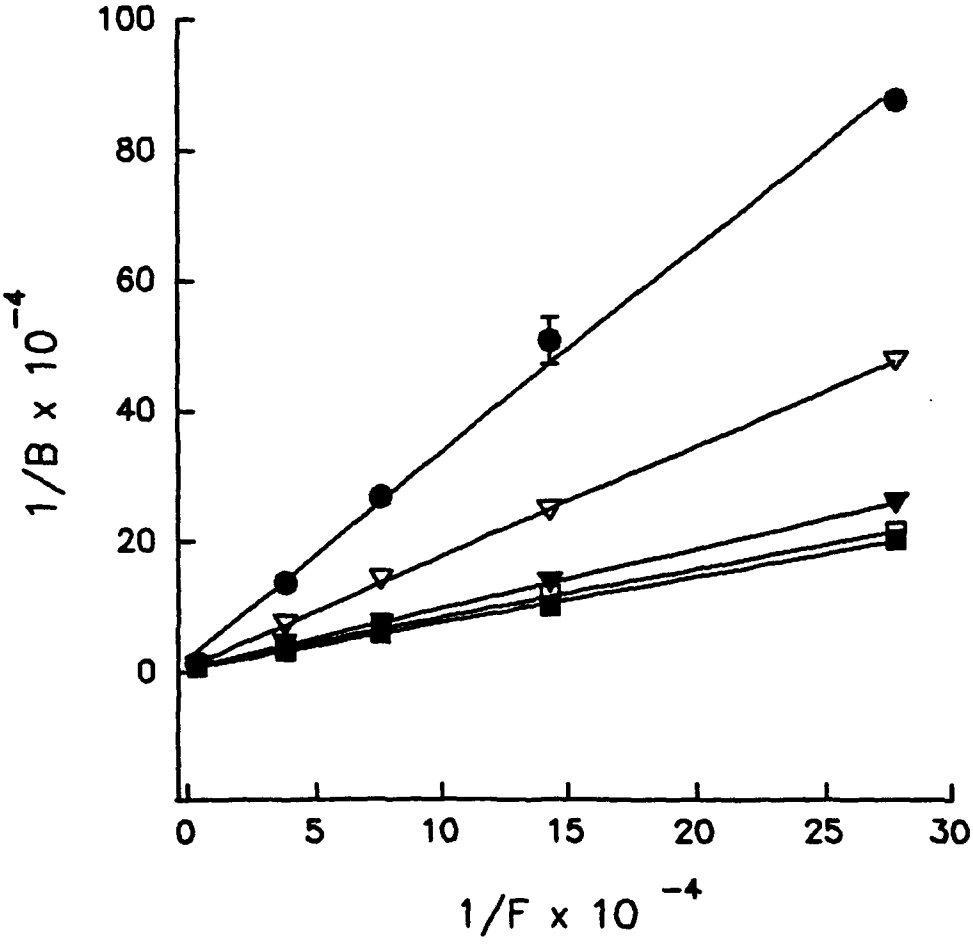


TABLE I

Pre-UV washes[1]

Number	Total (DPM)	Binding Nonspecific (DPM)	Specific (%)
0	29000	14000	53
1	27000	9000	67
2	27000	6000	79
3	24000	4000	83
4	17000	3000	82
5	12000	2000	86

[1]A number of washes prior to UV irradiation with buffer containing 1% BSA resulted in specific binding activity as shown. Unlabeled PbTx3-Pho was used at a concentration of 10 μM to assay nonspecific binding. The average error of the mean of duplicate determinations was 7%.

TASK IV. IDENTIFICATION OF PROTEIN COMPONENTS OF NEUROTOXIN RECEPTOR SITES 1 AND 5

The goal of this Task is to identify the protein components of neurotoxin receptor sites 1 and 5 by photoaffinity labeling and antibody mapping experiments. Our experiments to date have focussed primarily on neurotoxin receptor site 5 using a photoreactive, ^{3}H-labeled derivative of brevetoxin. These experiments have been carried out in collaboration with Dr. Daniel Baden of the University of Miami.

Experimental Procedures

Purification of photolabeled sodium channels from synaptosomes. The method of purification was based on the procedure of Hartshorne and Catterall (1984) with modifications as required for the smaller amount of starting material and the radioactivity of the starting material. A covalent labeling experiment using 5 nM [^{3}H]PbTx3-Pho was performed as described under Task III above. The solution was centrifuged, and the pellet was resuspended in solubilization solution (Hartshorne et al., 1980). Over a 30 min period, twelve 0.2 ml aliquots of 4% Triton X-100 were added with stirring at 0° C to double the sample volume, resulting in a final concentration of 2% Triton X-100. Unsolubilized membrane fragments were sedimented at 110,000 x g for 60 min. The supernatant solution was harvested and further purified using gel filtration.

DEAE-Sephadex A-25 (2.75 x 23 cm column with a 65 ml packed gel volume) was equilibrated with a buffer containing 120 mM KCl, 20 mM histidine HCl (adjusted to pH 6.5 with Tris base), 10 mM $CaCl_2$, 1.0% Triton X-100 and 0.12% egg phosphatidylcholine. The membrane extract was recirculated through the equilibrated column (see equilibration protocol below) at least 3 times. The resin was washed with at least 3 column volumes of equilibration buffer. The column was connected to a fraction collector and the sample was eluted from the gel with a buffer identical to the equilibration buffer but with an increase in the KCl concentration to 1 M. Aliquots of the one ml fractions were assayed for protein and radioactivity. Fractions containing [^{3}H]PbTx3-Pho were pooled for further purification.

The eluate from the ion exchange column was further purified on 9.5 ml packed volume of WGA-Sepharose in a 0.9 x 15 cm column. The lectin was equilibrated as described below for column regeneration. Pooled fractions from the DEAE Sephadex A-25 column were allowed to pass through the lectin column 3 times by gravity flow. The resin was washed with 50 column volumes of buffer III containing 0.15 M NaCl, 50 mM HEPES, 0.1% Triton X-100, pH 7.6. The sample was eluted from the column with buffer II (0.15 M NaCl, 50 mM HEPES, 0.1% Triton X-100, 0.3 M N-acetyl glucosamine, pH 7.6). One-half ml fractions were assayed for protein and radioactivity.

A typical purification of covalently labeled sodium channels by these procedures is illustrated in Table I.

Cleavage of photolabeled sodium channels by trypsinization and determination of ^{3}H-labeled peptide size by SDS-PAGE. PbTx3-Pho-labeled sodium channel, which had been purified through the WGA-Sepharose step, was dialyzed extensively at 4° C against distilled H_2O and then lyophilized. The PbTx3-Pho-labeled sodium channel was resuspended in a volume of Buffer S (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) which resulted in approximately 2000 DPM per 25 µl. TPCK-trypsin was added to give a final concentration of 10 mg/ml. The solution was incubated for the designated time and the reaction stopped by adding an equal volume of SDS sample buffer containing 2-mercaptoethanol (Laemmli, 1970). Samples were incubated at 100° C for 2 min, allowed to cool and subjected to SDS-PAGE and gel slicing as described above. TPCK-trypsin, which cleaves specifically at lysine and arginine residues, was stored frozen in small aliquots of Buffer S to assure that an identical concentration and lot number of the enzyme was used for each experiment.

Anti-peptide antibodies. Anti-peptide antibodies were prepared as described under Task I above. Rabbit antisera containing anti-sodium channel antibody were purified using protein A-Sepharose chromatography. The column was prepared by pre-equilibrating 500 mg protein A-Sepharose overnight at room temperature in 0.1 M sodium phosphate buffer, pH 8. The gel was slowly decanted into a small column. One ml serum was diluted with 1 ml binding buffer containing 0.15 M NaCl, 20 mM sodium phosphate, pH 8, and slowly loaded onto the column. The resin was washed with 6 ml binding buffer, then eluted with 0.1 M glycine, pH 3. One-half ml fractions were collected. Protein was monitored at 280 nm in a spectrophotometer. Peak protein fractions were pooled and concentrated with Amicon 30,000 MW cutoff centrifugation filters. The sample pH was adjusted to 7.4 with Tris base prior to use in immunoprecipitation studies. Protein A-Sepharose columns were regenerated as described above.

Immunoprecipitation. This method is a modification of the procedure described by Tejedor and Catterall (1988). WGA-Sepharose purified PbTx3-Pho-labeled sodium channel was resuspended in Buffer S to give a final activity of about 1000 DPM per 50 µl. One tenth final volume of TPCK-trypsin or Buffer S (control) was added and the sample digested for the appropriate time indicated in figure legends. After addition of 0.1% SDS, the sample was incubated at 100° C for 3 min, then cooled to room temperature by swirling on ice. Soybean trypsin inhibitor (Sigma) was added to a final concentration of 30 µg/ml and incubated for 5-10 min at 4° C. The following series of effectors were then added to give the indicated final concentrations: 1% Triton X-100, 150 mM NaCl, 0.5 mg/ml BSA. The appropriate sodium channel antibody was added to give a final concentration of 1.2 mg/ml. The reaction mixture was incubated with rotation at 4° C overnight.

Fifteen mg of protein A-Sepharose (150 µl of 100 mg/ml stock), swollen for 1 hr in TBS buffer (0.5 mg/ml BSA, 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) was added and incubated with rotation at 4° C for 45 min. The sample was centrifuged for 15 sec in a microfuge, the supernatant solutions discarded and the pellet resuspended in 1 ml TBS. This step was repeated 2 more times. The pellet was assayed for radioactivity.

Phosphorylation of WGA-purified sodium channel. This method was performed as described by Schmidt et al. (1985). Five hundred pmoles of sodium channel purified through the step of WGA-Sepharose (described above) were added to a solution of 100 µM ATP, 10 mM $MgCl_2$, 0.05% Triton X-100, 0.1 µg/pmol cAMP-dependent protein kinase, 0.25 µCi/pmol ^{32}P ATP and incubated at 0° C for 60 min. The reaction was stopped by adding EDTA to give a final concentration of 20 mM. The mixture was divided into 8 equal aliquots, then centrifuged in a microfuge for 45 sec through eight G-50 columns (2 ml volume packed in 3 ml syringes) pre-equilibrated with NET buffer. The column eluate was assayed for radioactivity and used in RIAs.

Radioimmunoassay. Varying concentrations of antibody were added to 0.1 M glycine, pH 7.4 and incubated with 5 pmol ^{32}P-labeled Na^+ channel in NET buffer (75 mM NaCl, 2.5 mM EDTA, 25 mM Tris, pH 7.4, 0.1% Triton X-100, 50 mM NaH_2PO_4, 20 mM KF). This mixture was incubated for 4-16 hrs at 4° C with rotation.

Five mg of protein A-Sepharose, swollen 1 hr in NET buffer, was added and the solution incubated for 45 min at 4° C with rotation. The sample was centrifuged at 4° C for 15 sec and the supernatant solution discarded. One ml NET buffer was added, the sample resuspended and centrifuged as before. This step was repeated two more times. The pellet was assayed for radioactivity.

Results

TPCK-trypsin digestion. In order to optimize specific antibody recognition of Na^+ channel peptides, determination of enzyme concentration and digestion time needed to obtain protein fragments having sizes similar to a subunit domains (approximately 70 kD) was necessary. In preparation for immunoprecipitation studies, the PbTx3-Pho NaCh was treated with varying

concentrations of TPCK-trypsin and filtered through polysulfone centrifugation filters (Millipore) to monitor the generation of appropriate peptide fragments. Estimates of TPCK-trypsin concentrations needed to give a peptide size useful for immunoprecipitation studies were made by SDS-PAGE. SDS gels containing typsinized PbTx3-Pho NaCh were sliced and the radioactivity associated with peptide fragments <46 kDa, 46-69 kDa, 69-200 kDa and >200 kDa was determined. A size range of peptides useful for immunoprecipitation studies resulted after a 1 to 60 min incubation with 10 μg/ml TPCK-trypsin (Fig. 1A). Treatment with 100 μg/ml TPCK-trypsin resulted in approximately 40% peptides smaller than 46 kD after only 1 min digestion (Fig. 1B). Treatment with 10 μg/ml was chosen as the condition for further studies.

Radioimmunoassay. Rabbit antibodies recognizing conserved peptide sequences from the four separate Na^+ channel α subunit domains were assayed for activity by RIA to confirm their relative potency for the immunoprecipitation experiments (Fig. 2). Antibodies to SP1, SP14, and SP29 all showed high affinity for their Na^+ channel recognition sequences, whereas this preparation of anti-SP35 was shown to have relatively weak affinity for its antigenic site. Results obtained using the latter antibody were not documented in this report due to low sample radioactivity.

Identification of photolabeled peptide fragments by immunoprecipitation. In the first immunoprecipitation experiment, the antibody to SP1 was incubated with intact (no trypsin treatment) PbTx3-Pho NaCh. This antibody showed significant recognition of the brevetoxin-linked Na^+ channel protein covalent conjugate over the preimmune serum control (Fig. 3). Although the level of radioactivity in the samples was small, the recognition by anti-SP1 was statistically significant ($p<0.05$). Increase of antibody protein to 1 mg/ml resulted in 30% greater immunoprecipitation of PbTx3-Pho-labeled sodium channel than the 0.1 mg/ml value (not shown). Tryptic digestion of the tritiated brevetoxin/Na^+ channel conjugate for 1, 10, or 60 min at 37° C followed by immunoprecipitation showed that only the domain IV antibody (anti-SP29) retained greater than 60% of its control binding activity at the final time point (Fig. 4A). This experiment was repeated using an additional domain IV antibody (anti-SP13) which recognizes a sequence on the same extracellular loop in domain IV as anti-SP29. Results from both experiments were similar (Fig. 4B), showing that both domain IV antibodies recognized a tryptic peptide covalently linked to the brevetoxin photoaffinity probe.

Discussion

Significance of brevetoxin binding to domain IV. Our initial results indicating brevetoxin binding to protein segments in domain IV are of great interest. Results on calcium channels have indicated that both phenylalkylamine and dihydropyridine calcium channel modulators bind to sequences in this domain. Dihydropyridines have effects on calcium channel gating which resemble in important respects the effects of brevetoxins on sodium channels. Moreover, at high concentrations, dihydropyridines are known to have effects on sodium channels themselves. It seems likely that general mechanisms of pharmacological modulation of the voltage-gated ion channels may emerge from studies of the receptors sites for pharmacological agents on these two related ion channels.

Future experiments. We will follow up these initial antibody mapping studies in two directions. We will use the existing photolabeling and antibody mapping methods to further define the peptide fragments of domain IV of the α subunit which are involved in brevetoxin binding. In addition, we will complete development of a new ^{125}I-labeled photoaffinity reagent which appears very promising in our current experiments. This new reagent will allow antibody mapping at substantially higher resolution because of its higher specific radioactivity.

Figure Legends

FIG. 1. SDS-PAGE analysis of PbTx3-Pho-labeled sodium channel treated with TPCK-trypsin. Gel lanes were cut into 4 mm slices, compared to molecular weight standards, and assayed for radioactivity as described in Experimental Procedures. Na^+ channel preparations were treated with 10 μg/ml (A) or 100 μg/ml (B) TPCK-trypsin at 37° C for the designated time. Standard molecular weight markers (see symbol legend) are myosin (200 kD), bovine serum albumin (69 kD), and ovalbumin (46 kD). Error bars, when present, show range of duplicate determinations.

FIG. 2. Radioimmunoassay of antibodies directed against Type IIA Na^+ channel sequences. A ^{32}P-labeled Na^+ channel preparation was incubated with rabbit antibodies directed against peptides located on each of the 4 channel domains. Protein was measured as A_{280}. Each point is a single determination.

FIG. 3. Immunoprecipitation of brevetoxin-labeled Na^+ channel. PbTx3-Pho-labeled sodium channel was incubated with either preimmune serum or anti-SP1. IgG protein concentrations, determined by absorbance at 280 nm, were 0.3 mg/ml (preimmune serum) and 0.1 mg/ml (antibody to SP1). Samples were counted for 20 min resulting in a 95% confidence level of ± 2% of the mean.

FIG. 4. Immunoprecipitation with anti-Na^+ channel antibodies with recognition sequences on 3 separate domains. A. PbTx3-Pho-labeled sodium channel (approximately 18 pmol protein per tube) was incubated with TPCK-trypsin at 37° C for the designated time and incubated with Na^+ channel antibodies (0.1 mg antibody protein per sample). Antigen/antibody complexes were immnoprecipitated with protein A-Sepharose using the procedure described in Experimental Procedures. Each point is a single determination. **B.** The experiment in panel A was repeated with 0.18 mg antibody protein per sample. An additional domain IV antibody (anti-SP13) was used. Total radioactivity for controls (100% total binding) was approximately 150 CPM. Blank CPM = 30. Samples were counted for 20 min resulting in a 95% confidence level of ± 2% of the mean.

FIGURE 1.

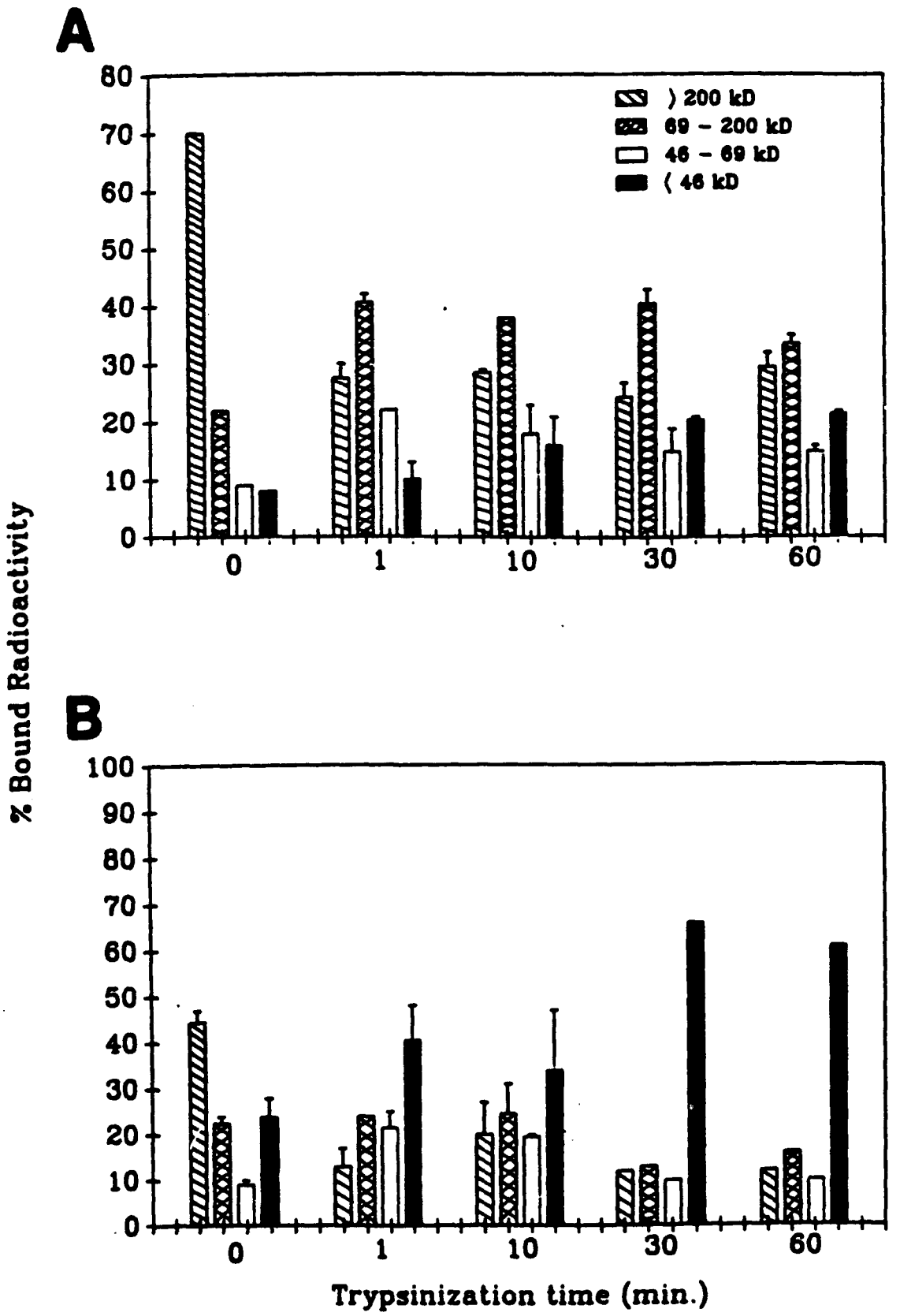

A
B
) 200 kD
69 – 200 kD
46 – 69 kD
(46 kD
% Bound Radioactivity
Trypsinization time (min.)
0
1
10
30
60

FIGURE 2.

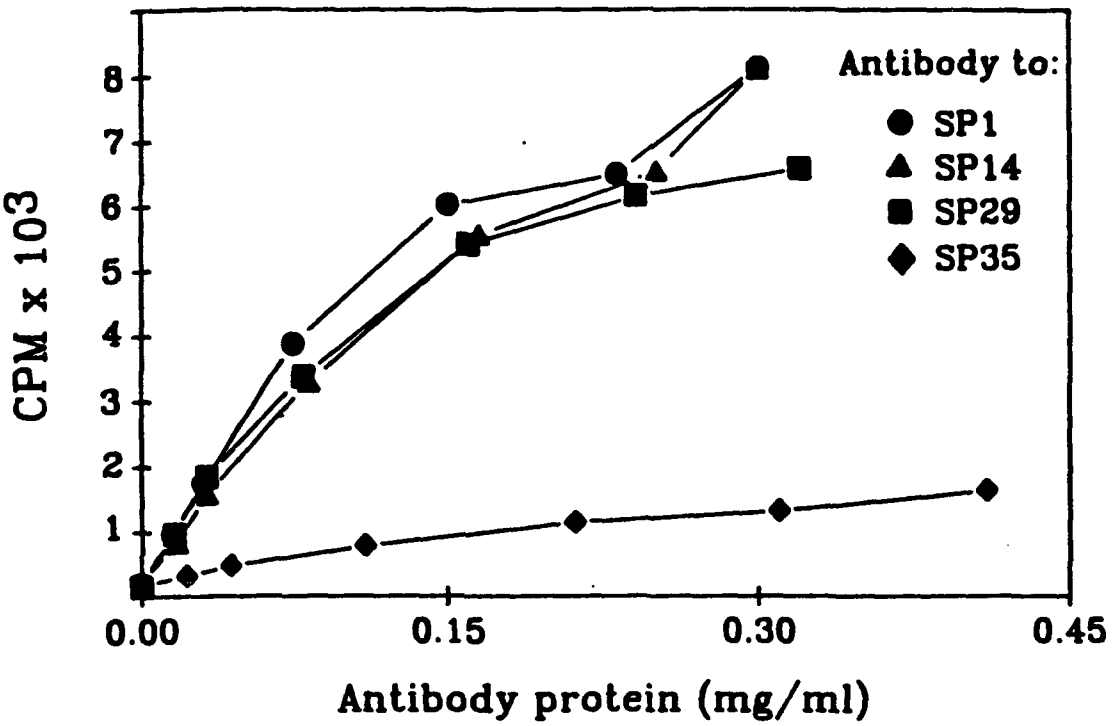

CPM x 10^3
8
7
6
5
4
3
2
1
0.00
0.15
0.30
0.45
Antibody protein (mg/ml)
Antibody to:
SP1
SP14
SP29
SP35

FIGURE 3.

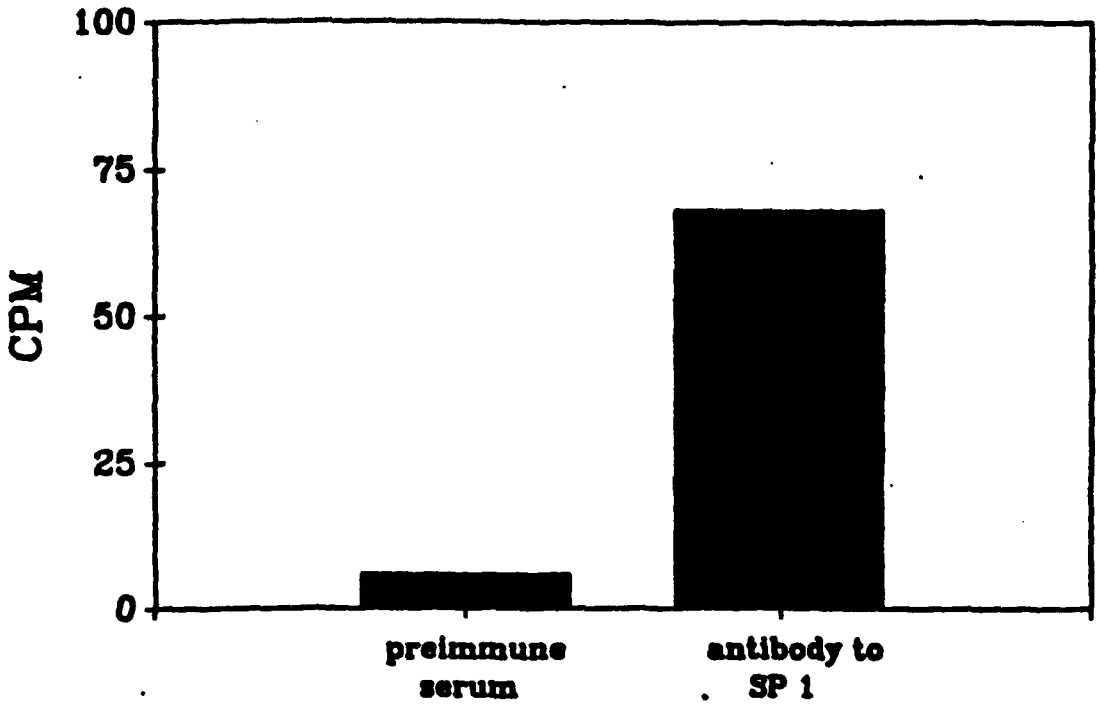


FIGURE 4.

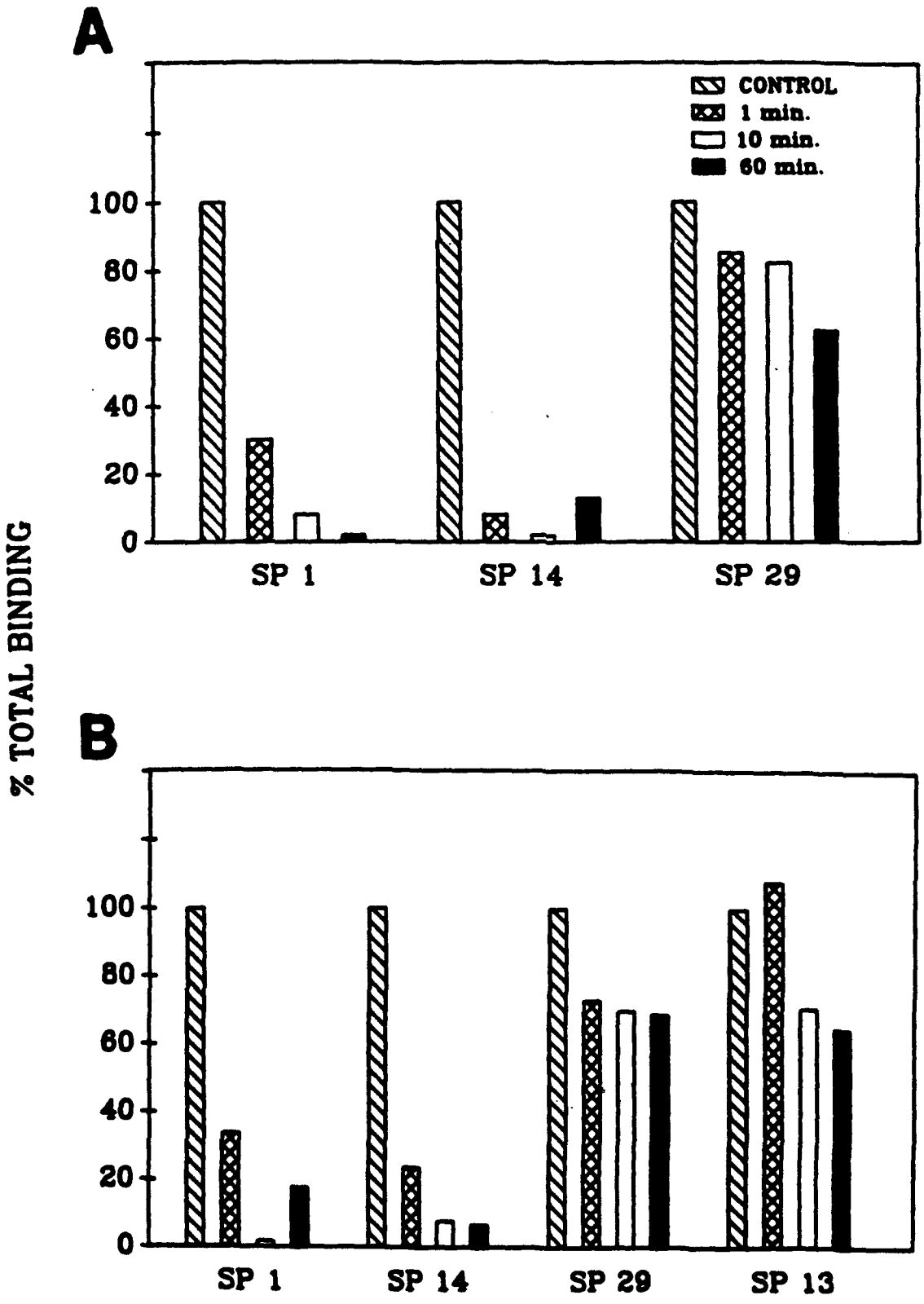

A
CONTROL
1 min.
10 min.
60 min.
100
80
60
40
20
0
SP 1
SP 14
SP 29
% TOTAL BINDING
B
100
80
60
40
20
0
SP 1
SP 14
SP 29
SP 13

TABLE I

Brevetoxin Receptor Purification[1]

Step	PbTx receptor		Protein		Activity	Purification
	pmol	%	*mg*	%	*pmol/mg*	*-fold*
Membrane extract	0.55	100.0	1.03 ± 0.04	100.0	0.55	1.0
DEAE-Sephadex	0.32 ± 0.04	29.6	0.20 ± 0.002	24.4	1.66 ± 0.2	3.0
WGA-Sepahrose	0.26 ± 0.04	24.0	0.07 ± 0.01	9.0	3.64 ± 0.1	6.6

[1]Receptor activity was measured as the specific radioactivity of the tritiated photoaffinity Na^+ derivative. Values are ± standard deviation, n = 3 where indicated.

TASK V. INHIBITION OF NEUROTOXIN BINDING TO RECEPTOR SITES 1 AND 5

Although we have not yet identified the protein components of neurotoxin receptor sites 1 and 5 by photoaffinity labeling and antibody mapping, work in other laboratories using site-directed mutagenesis methods has succeeded in identifying negatively charged amino acid residues that are required for high affinity binding of tetrodotoxin and saxitoxin at neurotoxin receptor site 1 (Terlau et al., 1991). In these experiments, neutralization of one or two negatively charged residues located just on the extracellular side of transmembrane segment S6 in each domain caused 1000- to 10000-fold reductions in the affinity of the sodium channel for tetrodotoxin and saxitoxin. We have examined whether antibodies that recognize amino acid segments adjacent to or including these amino acid residues are effective in preventing the high affinity binding of saxitoxin to sodium channels.

Experimental Procedures

Binding of [^{3}H]saxitoxin to sodium channels in synaptosomes was measured at 1 nM toxin using a vacuum filtration method as described by Catterall et al (1979). Binding of [^{3}H]saxitoxin to purified sodium channels was measured using a rapid gel filtration method as described by Catterall et al (1979). Synaptosomes and purified sodium channels were pre-incubated with the indicated antibodies for 4 hr at 4°C in binding medium before addition of [^{3}H]saxitoxin to the binding reaction.

Results

Synaptosomes were pre-incubated with approximately 7 μM of each antibody preparation to be tested. [^{3}H]saxitoxin was added and specific saxitoxin binding was measured as described under Experimental Procedures. None of the antibodies tested had a substantial effect on saxitoxin binding. In particular, antibodies against peptides SP28 and SP34 which contain amino acid residues that are essential for toxin binding had no significant effect (Fig. 1). This is a surprising result and suggests that the critical residues are inaccessible to the antibody for binding. The interaction of the antibody with the sodium channel must be mediated by the other adjacent amino acids in the peptide segment against which the antibody was made.

In order to improve the access of the antibody to the critical region of the sodium channel sequence, we tested the effects of the same panel of antibodies on saxitoxin binding to purified sodium channel solubilized in Triton X-100. Even under these conditions, we found only small or no effects of site-directed antibodies on saxitoxin binding (Fig. 2). Peptide SP42 was designed to include the amino acid residues that are essential for toxin binding to neurotoxin receptor site 1 in domain II (Task I, Fig. 1B). This peptide proved to be too hydrophobic for our usual antibody development techniques, so a new method for solubilization and immunization was developed (see Experimental Procedures under Task 1). Tests of the antibody against this peptide indicated that a small inhibition of toxin binding was caused by this antibody at 7 μM (Fig. 2). Nevertheless, this small level of inhibition at high antibody concentration is not a very encouraging result.

Overall, the results with antibody block of neurotoxin binding to receptor site 1 indicate that the critical residues for high affinity binding are not blocked when the polyclonal anti-peptide antibodies that we have developed to date are bound to the sodium channel.

Discussion

Future Experiments. Our standard protocol for preparation of anti-peptide antibodies involves synthesis of 18 to 20 residue peptides. We have found that this procedure is optimum for

production of antibodies that recognize the channel protein as well as the synthetic peptide antigen. Our polyclonal anti-peptide antibodies contain many different individual antibody species that recognize different segments and conformations of the peptide antigen. Many of these antibodies may be able to bind to the protein by interaction with residues that are not required for toxin binding and therefore may not be effective inhibitors of toxin binding. Moreover, if antibodies that recognize the critical residues are rare in the polyclonal population of IgG molecules, they may be prevented from reaching their recognition site by the binding of other antibody molecules to adjacent protein segments. To circumvent this problem, we plan to isolate the antibody molecules that recognize the critical amino acid residues specifically by affinity purification of the antibodies using short synthetic peptides immobilized on Sepharose beads. We will synthesize 5 to 7 residue peptides including two critical amino acid residues, covalently attach them to Sepharose beads, and purify antibodies by adsorbtion to them. We will then test these antibodies for inhibition of toxin binding. If these antibodies of more sharply restricted specificity are effective in blocking toxin binding, we will prepare monoclonal antibodies against the short peptides to provide even more specific reagents.

Figure Legends

FIG. 1. Effects of site-directed antibodies on saxitoxin binding to sodium channels in synaptosomes. Rat brain synaptosomes were pre-incubated with approximately 7μM of the indicated antibodies for 4 hr at 4°C and specific binding of [^{3}H]saxitoxin was measured as described under Experimental Procedures.

FIG. 2. Effects of site-directed antibodies on saxitoxin binding to purified sodium channels. Sodium channels solubilized in Triton X-100 and purified from rat brain were pre-incubated with approximately 7μM of the indicated antibodies for 4 hr at 4oC and specific binding of [3H]saxitoxin was measured as described under Experimental Procedures.

FIGURE 1.

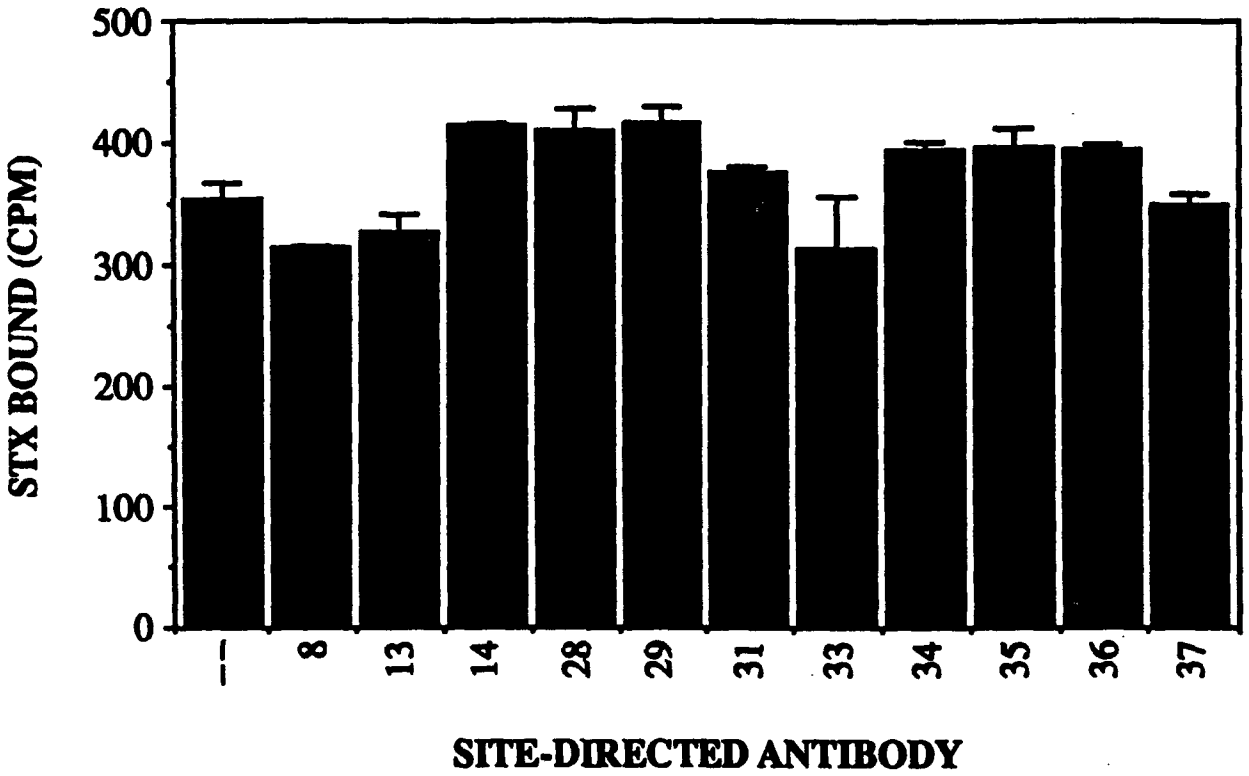
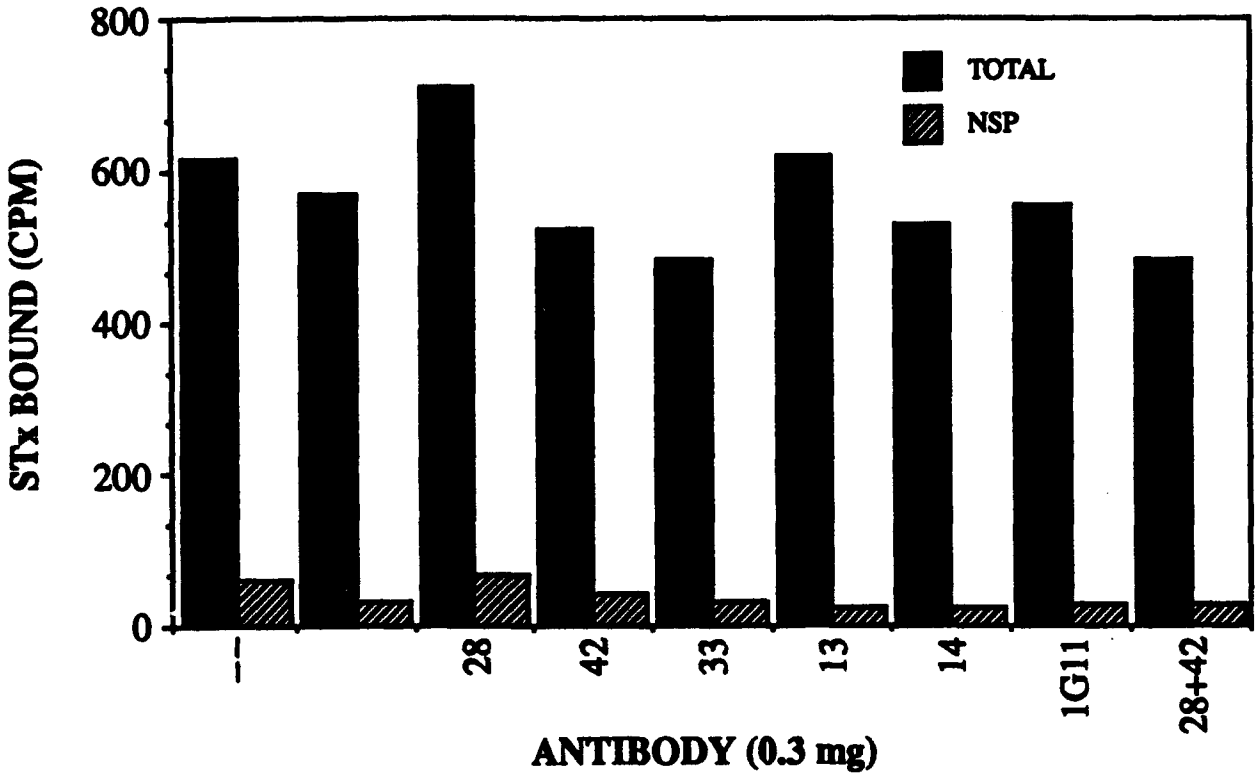

500
400
300
200
100
0
STX BOUND (CPM)
–
8
13
14
28
29
31
33
34
35
36
37
SITE-DIRECTED ANTIBODY

FIGURE 2.


800
600
400
200
0
STx BOUND (CPM)
TOTAL
NSP
--
28
42
33
13
14
1G11
28+42
ANTIBODY (0.3 mg)

REFERENCES

Baden, D.G., Mende, T.J., Lichter, W., and Wellham, L.L. (1981) *Toxicon* **19**:455-462.
Catterall, W.A. (1977) *J. Biol. Chem.* **252**:8660-8668.
Catterall, W.A., Morrow, C.S., and Hartshorne, R.P. (1979) *J. Biol. Chem.* **254**:11379-11387.
Costa, M.R., and Catterall, W.A. (1984) *J. Biol. Chem.* **259**:8210-8218.
De Jongh, K.S., Merrick, D.K., and Catterall, W.A. (1989) *Proc. Natl. Acad. Sci. USA* **86**:8585-8589.
De Jongh, K.S., Warner, C., Colvin, A.A., and Catterall, W.A. (1991) *Proc. Natl. Acad. Sci. USA* **88**:10778-10782.
El Ayeb, M., Bahraoui, E.M., Granier, C., and Rochat, H. (1986) *Biochemistry* **25**:6671-6677.
Feller, D.J., Talvenheimo, J.A., and Catterall, W.A. (1985) *J. Biol. Chem.* **260**:11542-11547.
Gordon, D., Merrick, D., Wollner, D.A., and Catterall, W.A. (1988) *Biochemistry* **27**:7032-7038.
Gordon,D., Merrick, D., Auld, V., Dunn, R., Goldin, A.L., Davidson, N., and Catterall, W.A. (1987) *Proc. Natl. Acad. Sci. USA* **84**:8682-8686.
Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981) *Pflügers Arch.* **391**:85-100.
Hartshorne, R., and Catterall, W.A. (1984) *J. Biol. Chem.* **259**:1667-1675.
Laemmli, U.K. (1970) *Nature* **227**:680-685.
Merrifield, R.B. (1963) *J. Am. Chem. Soc.* **85**:2149-2154.
Orth, D.N. (1979) in *Methods of Hormone Radioimmunoassay*, eds. Jaffe, B.M. & Behrman, H.R. (Academic, New York), pp. 245-284.
Poli, M.A., Mende, T.J., and Baden, D.G. (1986) *Mol. Pharmacol.* **30**:129-135.
Schmidt, J., Rossie, S., and Catterall, W.A. (1985) *Proc. Natl. Acad. Sci. USA* **82**:4847-4851.
Tamkun, M.M., Talvenheimo, J.A., and Catterall, W.A. (1984) *J. Biol. Chem.* **259**:1676-1688.
Tejedor, F.J., and Catterall, W.A. (1988) *Proc. Natl. Acad. Sci. USA* **85**:8742-8746.
Terlau, H., Heinemann, S.H., Stuehmer, W., Pusch, M., Conti, F., Imoto, K., and Numa, S. (1991) *FEBS Lett.* **293**:93-96.
Thomsen, W.J., and Catterall, W.A. (1989) *Proc. Natl. Acad. Sci. USA* **86**:10161-10165.
West, J.W., Scheuer, T., Maechler, L., and Catterall, W.A. (1992) *Neuron* **8**:59-70.